Remarks

Applicant respectfully requests reconsideration. Claims 12, 17, 21, 22, 31, 36, 40, 71 and 72 were previously pending in this application. Applicant has amended claims 12, 21, 22 and 40 to correct minor informalities in the claims, without prejudice or disclaimer. As a result, claims 12, 17, 21, 22, 31, 36, 40, 71 and 72 remain pending for examination. No new matter has been added.

Objection to the Oath/Declaration

The Examiner objected to the declaration as defective because it does not identify the city and state or city and country of each inventor. Applicant submits herewith a supplemental Application Data Sheet with the requested information, and respectfully requests reconsideration of the objection.

Objections to the Specification

The Examiner objected to the specification for two reasons. First, the title of the application was asserted to be not descriptive. Applicant has amended the title. Second, the drawings were objected to. Applicant has amended the description of Figure 5. Applicant respectfully requests reconsideration of the objections to the specification.

Objections to the Claims

1. The Examiner objected to claims 12, 21, 31, 40, 71 and 72 for the recitation of "LKB1". Applicant respectfully requests reconsideration. The term LKB1 is what this gene and its protein product are known as in the art. The gene also is known by synonyms such as STK11 and PJS,

while the protein product of this gene is also known by synonyms such as serine/threonine kinase 11 and NY-REN-19. See, e.g., http://www.uniprot.org/uniprot/Q15831. To Applicant's knowledge, LKB1 is not an abbreviation; public databases of gene names do not include a different, "full" version of the gene or protein name corresponding to LKB1.

2. The Examiner objected to claims 12 and 22 for the recitation of the parenthetical phrases "(cells of)" and "(cells)", respectively. Applicant has amended these claims and respectfully requests reconsideration.

Rejections Under 35 U.S.C. § 112, Second Paragraph

The Examiner rejected claims 12, 17, 21, 22, 31, 36, 40, 71 and 72 under 35 U.S.C. § 112, second paragraph as indefinite. Applicant respectfully requests reconsideration.

[a] The Examiner asserted that the term "reduced...LKB1 activity" is indefinite. Applicant respectfully disagrees. According to MPEP 2173.02, the essential inquiry pertaining to this requirement is whether the claims set out and circumscribe a particular subject matter with a *reasonable* degree of clarity and particularity. Definiteness of claim language must be analyzed in light of the content of the specification, the teachings of the prior art, and the claim interpretation that would be given by a person of ordinary skill in the art at the time the invention was made.

Respectfully, it is "[o]nly when a claim remains insolubly ambiguous without a discernible meaning after all reasonable attempts at construction" that the claim should be considered indefinite. MPEP 2173.02, citing Metabolite Labs., Inc. v. Lab. Corp. of Am. Holdings, 370 F.3d 1354, 1366, 71 USPQ2d 1081, 1089 (Fed. Cir. 2004). It plainly is not the case that the instant claims are "insolubly ambiguous", since the person of ordinary skill in the art, having a high degree of skill, would understand with a reasonable degree of clarity and particularity what is meant by "reduced...LKB1 activity".

[b] The Examiner rejected claims 12 and 31, and dependent claims, for allegedly being incomplete for omitting essential elements, citing MPEP 2172.01. Specifically, the Examiner stated: "it would appear that the cell must express AMPK in order to achieve promoting apoptosis. In the absence of AMPK, it is unclear as to how a skilled artisan is to achieve promoting apoptosis with a compound that activates AMPK." Office Action at page 5. Applicant respectfully traverses the rejection.

It is not true that a cell *must* express AMPK, since one way to increase AMPK activity is to induce its expression. This is clearly stated in the specification, such as at page 17, lines 11-13:

> Any method for increasing LKB1 or AMPK activity will be useful in the treatment of disorders. LKB1 or AMPK activity can be increased by pharmacological activators of the enzyme activity or its expression.

Therefore the Examiner is incorrect in stating that the claims are incomplete for omitting essential elements.

[c] The Examiner noted that claims 21 and 40 lack antecedent basis for the term "the mutation". Applicant has amended these claims as suggested by the Examiner, thereby obviating this aspect of the rejection.

Rejections Under 35 U.S.C. § 112, First Paragraph

1. The Examiner rejected claims 12, 17, 21, 22, 31, 36 and 40 under 35 U.S.C. § 112, first paragraph as allegedly failing to comply with the written description requirement. Specifically, the Examiner objected to the description of the genus of compounds that activate AMPK as recited in the claims. Applicant respectfully traverses the rejection and requests reconsideration.

It is well established in the law that the specification need not repeat information that is well known in the art for the specification to provide an adequate written description of the claimed invention. This is stated, for example, in MPEP 2163 II.A.2.: "Information which is

well known in the art need not be described in detail in the specification." (citing Hybritech, Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1379-80, 231 USPQ 81, 90 (Fed. Cir. 1986)). See also MPEP 2163 II.A.3.(a), citing Capon v. Eshhar, 418 F.3d 1349, 1357, 76 USPQ2d 1078, 1085 (Fed. Cir. 2005).

MPEP 2163 II.A.3.(a) further states that the description needed to satisfy the written description requirement "varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence." Citing Capon v. Eshhar, 418 F.3d at 1357, 76 USPQ2d at 1084.

Indeed, MPEP 2163 II.A.2 acknowledges that there is, generally, "an inverse correlation between the level of skill and knowledge in the art and the specificity of disclosure necessary to satisfy the written description requirement." The review of the specification to determine if the description is adequate is "conducted from the standpoint of one of skill in the art at the time the application was filed (see, e.g., Wang Labs. v. Toshiba Corp., 993 F.2d 858, 865, 26 USPQ2d 1767, 1774 (Fed. Cir. 1993)) and should include a determination of the field of the invention and the level of skill and knowledge in the art." MPEP 2163 II.A.2.

At the time of filing of the application, the skilled person was familiar with a variety of compounds that increase AMPK activity. For example, the following is a non-exhaustive list of examples of articles describing activators of AMPK (in addition to those described in the specification) known to the skilled person at the time of the filing of this application. The listed references disclose compounds that vary widely with respect to both structure and mechanism of action, and include small organic molecules, peptides, polypeptides, nucleic acids, and fatty acids.

- Levine et al. (J Biol Chem. 2007; 282(28):20351-64. Epub May 22, 2007, copy enclosed) shows that siRNA-mediated knockdown of caveolin-1 significantly enhanced AMPK phosphorylation.
- Fisslthaler and Fleming (Circ Res. 2009 Jul 17;105(2):114-27, abstract enclosed) is a review article that describes that "the activity of the AMPK in endothelial cells can be

regulated by stimuli that affect cellular ATP levels, such as hypoxia as well as by fluid shear stress, Ca(2+)-elevating agonists, and hormones such as adiponectin." (see e.g., the abstract.)

- Viollet et al. (Front Biosci. 2009; 14: 3380–3400, copy enclosed) is a review article that describes that physical exercise; AICAR and other compounds metabolized to AMP; two major classes of antidiabetic drugs (biguanides and thiazolidinediones); and insulin sensitizing adipokines (e.g., leptin, adiponectin and resistin) are known to activate AMPK.
- Cool et al. (Cell Metab. 2006 Jun;3(6):403-16, abstract enclosed) describes the identification of a thienopyridone family of AMPK activators, including the compound A-769662.
- Hutchinson et al. (Cell Signal. 2007 Jul;19(7):1610-20. Epub Feb 21, 2007, abstract enclosed) shows that NADPH oxidase inhibitors such as diphenylene iodonium (DPI) and apocynin activate AMPK.
- Ruderman et al. (Diabetes. 2006 Dec;55 Suppl 2:S48-54, copy enclosed) shows that interleukin-6 increases AMPK activity.
- Wang et al. (Biochem Biophys Res Commun. 2007 Jan 12;352(2):463-8. Epub Nov 16, 2006, abstract enclosed) describes that palmitate activates AMPK.
- Sun et al. (Circulation. 2006 Dec 12;114(24):2655-62. Epub Nov 20, 2006, copy enclosed) shows that statins activate AMPK.
- Brusq et al. (J Lipid Res. 2006 Jun;47(6):1281-8. Epub Feb 28, 2006, copy enclosed) demonstrates that the alkaloid drug berberine activates AMPK.
- Ha et al. (Pharmacogenomics J. 2006 Sep-Oct;6(5):327-32. Epub Jan 17, 2006, abstract enclosed) shows that topiramate activates AMPK.

Therefore, there was ample knowledge of AMPK activators in the art at the time that the application was filed.

More generally, "[t]he fundamental factual inquiry is whether the specification conveys with reasonable clarity *to those skilled in the art* that, as of the filing date sought, applicant was in possession of the invention as now claimed." MPEP 2163 I.B., emphasis added (citing Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 1563-64, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991).

MPEP 2163.05 cites In re Herschler, 591 F.2d 693, 697, 200 USPQ 711, 714 (CCPA 1979) as holding that disclosure of "corticosteriod in DMSO was sufficient to support claims drawn to a method of using a mixture of a 'physiologically active steroid' and DMSO because 'use of known chemical compounds in a manner auxiliary to the invention must have a corresponding written description only so specific as to lead one having ordinary skill in the art to that class of compounds. Occasionally, a functional recitation of those known compounds in the specification may be sufficient as that description.' "

The Examiner has acknowledged that the specification contains a description of compounds of the genus recited in the claims, and that these are *representative species* of the genus. (Office Action at page 8.) The Examiner, however, appears to apply an inappropriately stringent standard in stating that "the disclosed representative species *fail to describe all members of the recited genus* of compounds. (Office Action at page 8, emphasis added.) This is not the standard of the law because "representative" species are not meant to include all species in a genus.

MPEP 2163.04 describes the burden on an examiner with regard to the written description requirement, and notes that there "there is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed," citing In re Wertheim, 541 F.2d 257, 262, 191 USPQ 90, 96 (CCPA 1976).

In view of the examples of activators of AMPK described in the specification, which were acknowledged by the Examiner to be representative species, and the knowledge of the skilled person, Applicant submits that the specification provides an adequate written description of the invention as claimed. Accordingly, Applicant respectfully requests that the Examiner withdraw the rejection of the claims as lacking an adequate written description.

2. The Examiner rejected claims 12, 17, 21, 22, 31, 36 and 40 under 35 U.S.C. § 112, first paragraph as allegedly failing to comply with the enablement requirement. Specifically, the Examiner found that the specification enabled the claims for the compounds recited at page 11,

lines 1-10, but did not enable the claims for using any compound that increases or activates AMPK.

Applicant respectfully traverses the rejection and requests reconsideration based on a full consideration of the factors supporting enablement of the claims.

The Examiner's rejection is based on the assertion that it would require undue experimentation for a skilled person to make and use the whole scope of the claimed invention. More particularly, on page 10 of the Office Action the Examiner stated that the specific guidance provided in the specification is limited to compounds that directly affect AMPK activity, and that the claims recite the use of a broader class of compounds that includes compounds that indirectly affect AMPK activity.

First, the understanding of the skilled person, who is highly skilled in this art, was not completely described by the Examiner. Applicant has provided above in the preceding section addressing the written description rejection examples of the wide variety of agents known in the art to activate AMPK. It is well established that the specification need not disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public. See MPEP 2164.05(a), citing In re Buchner, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); Hybritech, Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), cert. denied, 480 U.S. 947 (1987); and Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co., 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).

In addition, MPEP 2164.03 states that "[t]he scope of the required enablement varies inversely with the degree of predictability involved, *but even in unpredictable arts, a disclosure of every operable species is not required*." (emphasis added) As described above in the response to the written description rejection, Applicant has provided an extensive description of AMPK activators in the specification and the literature provides additional extensive examples of AMPK activators known to the skilled person at the time of the filing of this application that are widely variant with respect to both structure and mechanism of action.

Second, the Examiner acknowledges that methods of screening for compounds that activate or increase AMPK activity were known in the art. (Office Action at page 10.) This favors a finding that the experimentation to identify additional AMPK activators was not undue. MPEP 2164.01 states that the fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation, citing In re Certain Limited-Charge Cell Culture Microcarriers, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), aff'd. sub nom., Massachusetts Institute of Technology v. A.B. Fortia, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985)).

Therefore, the factors of state of the prior art, the level of skill and knowledge of the person of ordinary skill in the art, the presence of working examples, and the quantity of experimentation needed, as set forth in In re Wands, favor a finding that the claims are enabled. In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Accordingly, Applicant respectfully requests that the Examiner withdraw the rejection of the claims as not enabled.

Rejections Under 35 U.S.C. § 102

The Examiner rejected claims 31 and 40 under 35 U.S.C. § 102 as anticipated by Shen et al. (Clin. Cancer Res. 8:2085-2090, 2002; "Shen"). Applicant respectfully traverses the rejection.

The rejected claims recite methods for *promoting apoptosis* of cells having reduced or absent LKB1 activity. As is well known in the art, apoptosis is the process of programmed cell death. In contrast to apoptosis, the outcome of over-expression of LKB1 is reported by Shen to be *growth inhibition* mediated though G_1 cell cycle arrest. (See Shen at page 2089, left column, first paragraph.) Shen does not, however, disclose a method for promoting apoptosis of cells having reduced or absent LKB1 activity, comprising contacting the cells with a compound that is an activator of AMP-activated protein kinase (AMPK) as recited in the claims.

Therefore, Shen does not teach all of the elements of the claimed invention. Accordingly, Applicant respectfully requests reconsideration of the rejection of claims 31 and 40 under 35 U.S.C. § 102.

Rejections Under 35 U.S.C. § 102/103

1. The Examiner rejected claims 12, 17, 21, 31, 36, 40 and 71-72 under 35 U.S.C. § 102, as anticipated by Dilman et al. (Arch. Geschwulstforch 48:1-8, 1978; "Dilman1") or Dilman et al. (Gerontology 26:241-246, 1980; "Dilman2") as evidenced by Shen (as above) and Zhang et al. (Am. J. Physiol. Circ. Physiol. 293:H457-H456, 2007). Applicant respectfully traverses the rejection.

The Examiner apparently recognized the failure of Dilman1 and Dilman2 to teach that phenformin has an effect on cancers characterized by reduced or absent LKB1 activity, because the Examiner cited the Shen reference as evidencing that LKB1 expression is reduced in breast cancer. Respectfully, Shen does not evidence any such fact. Shen teaches that two specific breast cancer cell lines, MDA-MB-435 and MDA-MB-231, lack LKB1 expression (see Fig. 1). However, Shen also teaches that the breast cancer cell line MCF7 does not have reduced LKB1 expression (see Fig. 1). In addition, Shen shows that LKB1 expression is variable in breast cancer samples (see Fig. 5 and Table 2).

Applicant notes that the MDA-MB-435 cells have been shown more recently to be not of breast cancer origin, but rather to have been derived from M14 melanoma cells. See Rae et al. (Breast Cancer Res Treat. 2007 Jul;104(1):13-9. Epub Sep 27, 2006, abstract enclosed), which states in the conclusion of the abstract: "All currently available stocks of MDA-MB-435 cells are derived from the M14 melanoma cell line and can no longer be considered a model of breast cancer." Indeed, Rae et al. refer to a 2000 Nature article by Ross et al. that cast doubt on the breast cancer origin of these cells well before the publication of the Shen article.

The MPEP makes it clear that if a second reference is used as extrinsic evidence to fill in a gap in the allegedly anticipatory reference, "such evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill." MPEP 2131.01 III (emphasis added), citing Continental Can Co. USA v. Monsanto Co., 948 F.2d 1264, 1268, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991). Here, Shen only teaches that *certain* breast cancer cells and cell lines, but *certainly not all* breast cancer cells or cell lines, have reduced expression of LKB1. Thus Shen does not make clear that the missing descriptive matter is necessarily present, as is required under the law. MPEP 2131.01 III. Accordingly, the Examiner's use of Shen, to show that a characteristic of breast cancer cells that not disclosed in the Dilman references is inherent in such cells, is not effective to support the rejection of the claims.

Accordingly, Applicant respectfully requests reconsideration of the rejection of claims 12, 17, 21, 31, 36, 40 and 71-72 under 35 U.S.C. § 102.

2. The Examiner rejected claim 22 under 35 U.S.C. § 102, as anticipated by Dilman1 or Dilman2 as evidenced by Shen (as above) and Zhang (as above) and as further evidenced by Caraci et al. (Life Sci. 74:643-650, 2003; "Caraci"). The Examiner asserts that Caraci teaches that phenformin induces apoptosis of cancer cell lines. Applicant respectfully traverses the rejection.

First, Applicant submits that the cited references do not anticipate the claims for the same reasons set forth in the traversal above of the rejection made based on Dilman1 or Dilman 2.

Second, claim 22 recites a method that *further comprises* subjecting the cancer of the subject or cells thereof to a cell death stimulus. This means that the claim recites two steps: (1) administering to a subject having a cancer characterized by reduced or absent LKB1 activity an effective amount of a compound that increases AMPK activity, and (2) subjecting the cancer of the subject or cells thereof to a cell death stimulus. This combination treatment differs from a single treatment, e.g., phenformin, as is clear from the specification, which states: "These results

offer the provocative suggestion of a potential therapeutic window in which LKB1-deficient tumor cells might be acutely sensitive to AMP analogues *or sensitized to cell death by other stimuli if treated in combination with AMPK activators*." (Emphasis added, page 37, lines 5-7.)

Accordingly, Applicant respectfully requests reconsideration of the rejection of claim 22 under 35 U.S.C. § 102.

Rejections Under 35 U.S.C. § 103

The Examiner rejected claims 12, 17, 21, 22, 31, 36, 40 and 71-72 under 35 U.S.C. § 103, as unpatentable over the combination of Dilman1, Dilman2 and Dilman et al. (Cancer Lett. 7:357-361, 1979; "Dilman3") as evidenced by Shen (as above), Zhang (as above) and Caraci (as above). Applicant respectfully traverses the rejection.

The instant application describes, for the first time, that there is a link between LKB1 and AMPK, and that there is a particular benefit in increasing AMPK activity for tumors having reduced or absent LKB1 activity. This understanding and benefit were completely unexpected and surprising, as is described in the specification (see, e.g., page 2, lines 19-27; page 10, lines 27-30; page 17, lines 4-10). The unexpected results recited in the specification and embodied in the claims support a finding of non-obviousness, at least because the skilled person could not have had a reasonable expectation of success in making the claimed invention without the knowledge of the particular benefit in increasing AMPK activity for tumors having reduced or absent LKB1 activity.

As to the rejection of the claims as a whole, Applicant submits that the three Dilman references do not provide all of the elements of the claimed invention, as was detailed above for Dilman1 and Dilman2 in the responses to the anticipation rejections. Dilman1, Dilman2 and Dilman3 individually and in combination fail to teach that phenformin has an effect on cancers characterized by reduced or absent LKB1 activity. For each of these references, the Examiner cites the Shen reference as evidencing that LKB1 expression is reduced in breast cancer.

Respectfully, Shen does not evidence any such fact. As already discussed above, Shen teaches that there is variability in LKB1 expression in breast cancer cell lines and in breast cancer samples.

The Examiner asserts that Dilman3 teaches that phenformin inhibited growth of several types of cancers and potentiated the antitumor effect of anticancer agents, cyclophosphamide and hydrazine sulfate. However, there is no teaching or suggestion in Dilman3 that tumors that have reduced or absent LKB1 activity would obtain a particular benefit from phenformin. Therefore, Dilman3 does not supply elements missing from the combination of Dilman1 and Dilman2, and therefore the combination of Dilman1, Dilman2 and Dilman3 does not have a scope and content that renders the claimed invention obvious.

The feature of reduced or absent LKB1 activity is not a disclosed or inherent feature of the tumors treated in the Dilman references. Moreover, the combination of Dilman1, Dilman2 and Dilman3 does not teach or suggest, in any way, administering a compound that increases AMPK activity to a subject having a cancer characterized by reduced or absent LKB1 activity, or that there would be an unexpectedly increased therapeutic benefit in subjects that have a cancer characterized by reduced or absent LKB1 activity. Nor does the combination of references teach or suggest promoting apoptosis of cells having reduced or absent LKB1 activity by contacting the cells with a compound that is an activator of AMPK.

Therefore, the skilled person would not have a reasonable expectation of success in carrying out the claimed invention based on the teachings of the combination of Dilman1, Dilman2 and Dilman3 because the skilled person is not taught to administer to a subject having a cancer characterized by reduced or absent LKB1 activity an effective amount of a compound that increases AMPK activity, or to promote apoptosis of cells having reduced or absent LKB1 activity by contacting the cells with a compound that is an activator of AMPK.

There was no recognition in the cited prior art that compounds that increase AMPK activity would be useful in the claimed methods. To suggest that the cited combination of prior art provide a reason to administer to a subject having a cancer characterized by reduced or absent

LKB1 activity an effective amount of a compound that increases AMPK activity, or to promote apoptosis of cells having reduced or absent LKB1 activity by contacting the cells with a compound that is an activator of AMPK, is based purely on hindsight, which is impermissible. See MPEP 2142, which states that knowledge of an applicant's disclosure (hindsight) must be put aside in reaching a determination of whether the claimed invention as a whole would have been obvious at that time to the skilled person; and that such hindsight must be avoided.

Therefore, the combination of references cited by the Examiner does not render the claimed invention obvious because the combination does not teach or suggest all of the elements of the claimed invention, because it is not obvious to try to modify the teachings of the cited prior art to address the particular advantages of treating cancer characterized by reduced or absent LKB1 activity, and because the skilled person would not have had a reasonable expectation of success in doing so.

Accordingly, Applicant respectfully requests reconsideration of the rejection of claims 12, 17, 21, 22, 31, 36, 40 and 71-72 under 35 U.S.C. § 103.

CONCLUSION

A Notice of Allowance is respectfully requested. The Examiner is requested to call the undersigned at the telephone number listed below if this communication does not place the case in condition for allowance.

If this response is not considered timely filed and if a request for an extension of time is otherwise absent, Applicant hereby requests any necessary extension of time. If there is a fee occasioned by this response, including an extension fee, that is not covered by an enclosed check, please charge any deficiency to Deposit Account No. 23/2825.

Respectfully submitted,

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Agonist-modulated Regulation of AMP-activated Protein Kinase (AMPK) in Endothelial Cells

*EVIDENCE FOR AN AMPK → Rac1 → Akt → ENDOTHELIAL NITRIC-OXIDE SYNTHASE PATHWAY**

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The endothelial isoform of nitric-oxide synthase (eNOS), a key determinant of vascular homeostasis, is a calcium/calmodulin-dependent phosphoprotein regulated by diverse cell surface receptors. Vascular endothelial growth factor (VEGF) and sphingosine 1-phosphate (S1P) stimulate eNOS activity through Akt/phosphoinositide 3-kinase and calcium-dependent pathways. AMP-activated protein kinase (AMPK) also activates eNOS in endothelial cells; however, the molecular mechanisms linking agonist-mediated AMPK regulation with eNOS activation remain incompletely understood. We studied the role of AMPK in VEGF- and S1P-mediated eNOS activation and found that both agonists led to a striking increase in AMPK phosphorylation in pathways involving the calcium/calmodulin-dependent protein kinase kinase β. Treatment with tyrosine kinase inhibitors or the phosphoinositide 3-kinase inhibitor wortmannin demonstrated differential effects of VEGF *versus* S1P. Small interfering RNA (siRNA)-mediated knockdown of AMPKα1 or Akt1 impaired the stimulatory effects of both VEGF and S1P on eNOS activation. AMPKα1 knockdown impaired agonist-mediated Akt phosphorylation, whereas Akt1 knockdown did not affect AMPK activation, thus suggesting that AMPK lies upstream of Akt in the pathway leading from receptor activation to eNOS stimulation. Importantly, we found that siRNA-mediated knockdown of AMPKα1 abrogates agonist-mediated activation of the small GTPase Rac1. Conversely, siRNA-mediated knockdown of Rac1 decreased the agonist-mediated phosphorylation of AMPK substrates without affecting that of AMPK, implicating Rac1 as a molecular link between AMPK and Akt in agonist-mediated eNOS activation. Finally, siRNA-mediated knockdown of caveolin-1 significantly enhanced AMPK phosphorylation, suggesting that AMPK is negatively regulated by caveolin-1. Taken together, these results suggest that VEGF and S1P differentially regulate AMPK and establish a central role for an agonist-modulated AMPK → Rac1 → Akt axis in the control of eNOS in endothelial cells.

The AMP-activated protein kinase (AMPK)[2] is an evolutionarily conserved serine/threonine heterotrimeric kinase that was initially characterized as a "fuel gauge" modulating cellular energy flux in eukaryotic cells in response to changes in intracellular AMP levels (for a review, see Ref. 1). More recent studies have identified a broader role for AMPK in cellular homeostasis and signaling; AMPK is now known to be regulated by a family of upstream AMPK kinases, including the calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) (2, 3), and the tumor suppressor kinase LKB1 (4). After AMPK undergoes phosphorylation at the threonine 172 site in the activation loop of its catalytic α-subunit, the kinase is activated (1) and can mediate numerous energy-conserving cellular processes, such as promotion of glucose uptake and glycolysis (5), acceleration of mitochondrial biogenesis (6), and stimulation of fatty acid oxidation with concomitant inhibition of fatty acid synthesis via phosphorylation and inactivation of acetyl-CoA carboxylase (ACC) (7). In addition to phosphorylating ACC, AMPK also has been shown to phosphorylate the endothelial isoform of nitric-oxide synthase (eNOS) on serine 1179 in endothelial cells and cardiac myocytes (8). AMPK appears to be involved in the pathways of eNOS activation evoked by a variety of extracellular stimuli that modulate eNOS in endothelial cells, including metformin (9), adiponectin (10, 11), hypoxia (12), and hydroxymethylglutaryl-CoA reductase inhibitors (13). The activation of eNOS by AMPK has been implicated in many of the bioenergetic (14), angiogenic (12), and anti-inflammatory effects of AMPK in endothelial cells.

Despite the accumulating evidence linking AMPK with eNOS in the vascular endothelium, the molecular pathways involved in AMPK-mediated eNOS activation remain incompletely characterized. eNOS is also activated by the protein kinase Akt, a phosphoinositide 3-kinase (PI3K)-dependent effector that plays critical roles in numerous cellular responses, including angiogenesis and endothelial cell survival (15). A number of recent reports have studied both AMPK and Akt in the context of eNOS activation or nitric oxide release, but the relative role of these protein kinases remains controversial,

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[2] The abbreviations used are: AMPK, 5′-AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; BAEC, bovine aortic endothelial cell(s); eNOS, endothelial nitric-oxide synthase; CaMKKβ, calcium/calmodulin-dependent protein kinase kinase β; siRNA, small interfering RNA; PI3K, phosphoinositide 3-kinase; GSK3-β, glycogen synthase kinase-3-β; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; S1P, sphingosine 1-phosphate; ERK, extracellular signal-regulated kinase; FOV, field(s) of view; ANOVA, analysis of variance.

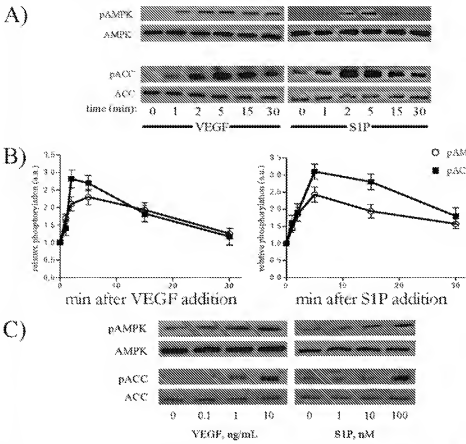

FIGURE 1. **Time course and dose response for VEGF- and S1P-mediated phosphorylation of AMPK and ACC.** *A*, results of immunoblots analyzed in BAEC lysates prepared from cells treated with VEGF (10 ng/ml) or S1P (100 nM) for the indicated times. Cell lysates were resolved by SDS-PAGE and probed using antibodies directed against phospho-AMPK, AMPK, phospho-ACC, and ACC, as indicated. The experiment shown is representative of four independent experiments that yielded similar results. *B*, results of densitometric analyses from pooled data, plotting the -fold increase of the degree of phosphorylation of AMPK at the times indicated, relative to the signals present in the unstimulated cells. Each data point represents the mean ± S.E. derived from four independent experiments. *C*, results of immunoblots analyzed in BAEC lysates prepared from cells treated with VEGF or S1P for 5 min at the indicated concentrations. Cell lysates were resolved by SDS-PAGE and probed using antibodies directed against phospho-AMPK, AMPK, phospho-ACC, and ACC. The experiment shown is representative of three independent experiments that give equivalent results.

with evidence for (16) and against (17) an AMPK/Akt interaction upstream of eNOS.

In addition to the uncertain relationship between AMPK and Akt, the eNOS-activating agonists that lead to AMPK phosphorylation are incompletely characterized, as are the pathways that connect these different phosphorylation pathways to eNOS activation and endothelial functional responses, including migration and tube formation. It remains unclear whether vascular endothelial growth factor (VEGF), an angiogenic polypeptide growth factor and potent eNOS agonist, promotes AMPK phosphorylation under normoxic conditions (12), and a recent study (18) that implicated AMPK in VEGF-mediated eNOS activation did not define the mechanisms involved in this response. Akt and eNOS are also potently activated by the platelet-derived lipid mediator sphingosine 1-phosphate (S1P) (19), but the phosphorylation of AMPK by S1P and the possible involvement of AMPK in S1P-mediated eNOS activation and cell motility have not been previously described. Both VEGF and S1P act in part by stimulating an influx of calcium into the endothelium (19, 20), thereby providing a route for AMPK activation that may depend upon CaMKK. The differential phosphorylation of AMPK by both VEGF and S1P therefore represents a plausible mechanism of eNOS regulation in endothelial cells.

Caveolae are plasmalemmal microdomains originally identified on the surface of endothelial and epithelial cells (21). The scaffolding/regulatory protein of caveolae, caveolin-1 (22, 23), is known to interact with and modulate the function of eNOS in endothelial cells (24). We have demonstrated that caveolin-1 negatively regulates the small GTPase Rac1 (25, 26), which in turn modulates the PI3K/Akt/eNOS pathway and regulates migration in endothelial cells (24, 27). It is therefore possible that AMPK regulates agonist-mediated eNOS activation and endothelial migration by interacting with caveolin-1 or Rac1 in endothelial cells.

In the present study, we provide evidence that the eNOS agonists VEGF and S1P differentially promote the phosphorylation of AMPK in vascular endothelial cells in distinct receptor-modulated pathways that involve tyrosine kinases and caveolin-1. Using pharmacological approaches as well as siRNA-mediated protein knockdown methodologies, we elucidate the molecular mechanisms of eNOS activation downstream of AMPK and identify a novel AMPK-Rac1-Akt pathway that functions as a critical determinant of eNOS activity as well as endothelial cell migration and tube formation in the vascular endothelium.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum was purchased from Hyclone (Logan, CT); all other cell culture reagents, media, and Lipofectamine 2000 transfection reagent were from Invitrogen. S1P and PP2 were from BioMol (Plymouth Meeting, PA). VEGF, genistein, wortmannin, cyclosporin, SB203580, STO-609, and Compound C were from Calbiochem. Polyclonal antibodies directed against phospho-AMPK (Thr^{172}), AMPK, phospho-ACC (Ser^{79}), ACC, phospho-eNOS (Ser^{1179}), phospho-Akt (Ser^{473}), Akt, phospho-GSK3-β (Ser^{9}), phospho-ERK1/2 (Thr^{202}/Tyr^{204}), and ERK1/2 were from Cell Signaling Technologies (Beverly, MA). Polyclonal Akt1 antibody was from Chemicon. eNOS monoclonal antibody, glycogen synthase kinase-3-β (GSK3-β) monoclonal antibody, and polyclonal caveolin-1 antibody were from BD Transduction Laboratories (Lexington, KY). The monoclonal antibody for VEGFR2 and polyclonal antibody specific for the β isoform of CaMKK were

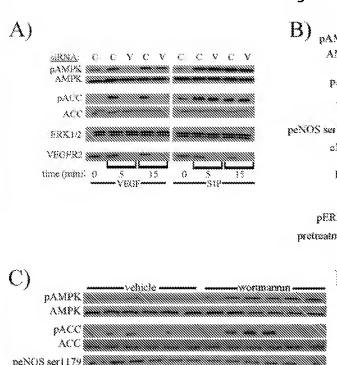

FIGURE 2. **Differential effects of siRNA-mediated VEGFR2 knockdown and protein kinase inhibitors on VEGF- and S1P-mediated AMPK and ACC phosphorylation.** *A*, an immunoblot of lysates prepared from BAEC that were transfected either with control (*C*) or VEGFR2 (*V*) siRNA and subsequently treated with VEGF (10 ng/ml) or S1P (100 nM) for the indicated times. Cell lysates were resolved on SDS-PAGE and analyzed in immunoblots probed with phospho-AMPK, AMPK, phospho-ACC, ACC, and VEGFR2-specific antibodies. The immunoblots in *B* show results of experiments exploring the effects of various inhibitors on S1P- and VEGF-mediated phosphorylation responses. BAEC were treated with VEGF, S1P, or their vehicle (Ø) for 5 min, and immunoblots of cell lysates were probed with the specific antibodies shown. Prior to agonist stimulation, cells were treated for 30 min with the tyrosine kinase inhibitor genistein (100 μM), the Src inhibitor PP2 (10 μM), the calcineurin inhibitor cyclosporin A (CsA; 100 nM), the p38 kinase inhibitor SB203580 (1 μM), or vehicle (−) as noted. In *C*, BAEC were pretreated with wortmannin (1 μM) or its vehicle and then treated with S1P for the indicated times. *D*, densitometric analysis of pooled data presenting the relative phosphorylation of AMPK after 5-min treatment with VEGF or S1P in the presence or absence of the inhibitors studied in the experiments shown in *B* and *C*. Each data point represents the mean ± S.E. derived from three independent experiments; for the vehicle-, cyclosporin A-, and SB203580-pretreated cells, the addition of VEGF and S1P induced a significant increase in AMPK phosphorylation ($p < 0.001$). *, $p < 0.05$ *versus* vehicle (ANOVA).

from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rac1 monoclonal antibody and Rac activation assay kit were from Upstate Biotechnology, Inc. (Temecula, CA). Super Signal substrate for chemiluminescence detection and secondary antibodies conjugated with horseradish peroxidase were from Pierce. Tris-buffered saline and phosphate-buffered saline were from Boston Bioproducts (Ashland, MA). Other reagents were from Sigma.

Cell Culture—Bovine aortic endothelial cells (BAEC) were obtained from Cell Systems (Kirkland, WA) and maintained in culture in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%, v/v) as described previously (19). Cells were plated onto 0.2% gelatin-coated culture dishes and studied prior to cell confluence between passages 5 and 9.

siRNA Design and Transfection—Our siRNA duplexes were designed on the basis of established characteristics of siRNA targeting constructs (28). All experimental oligonucleotides were purchased from Ambion (Austin, TX). We designed an AMPKα1 siRNA corresponding to bases 234–252 from the open reading frame of bovine AMPKα1: 5′-CCU CAA GCU UUU CAG GCA UdTdT-3′ (Ensembl Transcript ID: ENSBTAT00000000016). We also designed an Akt1 siRNA corresponding to bases 1325–1343 from the open reading frame of bovine Akt1 (5′-GGA CGU GUA CGA GAA GAA GdTdT-3′; Ensembl Transcript ID: ENSBTAG00000017636), a CaMKKβ siRNA from bases 585–603 of the open reading frame of bovine CaMKKβ (5′-GGU GCU GUC CAA AAA GAA AdTdT-3′; Ensembl Transcript ID: ENSBTAG00000010815), and an eNOS siRNA from bases 3948–3966 of the open reading frame of bovine eNOS (5′-CCU GAU CUC UAA AUC AUU CdTdT-3′; Ensembl Transcript ID: ENSBTAT00000007246). siRNA constructs targeting VEGFR2 (32), Rac1 (27), and caveolin-1 (24) have been described previously. A nonspecific control siRNA from Dharmacon (Lafayette, CO) was used as a negative control (5′-AUU GUA UGC GAU CGC AGA CdTdT-3′). BAEC were transfected with siRNA as described previously (24) and analyzed 48 h after transfection.

Drug Treatment and Immunoblotting—12–16 h prior to cell treatments, culture medium was changed to serum-free medium. VEGF and S1P were prepared as previously reported (24). Genistein, PP2, cyclosporin A, SB203580, wortmannin, STO-609, and Compound C were solubilized in Me_2SO and

kept at −20 °C; where indicated, 0.1% (v/v) Me_2SO was used as the vehicle control. After drug treatments, BAEC were washed with phosphate-buffered saline and incubated on ice for 20 min in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 2 mM Na_3VO_4, 1 mM NaF, 2 μg/ml leupeptin, 2 μg/ml antipain, 2 μg/ml soybean trypsin inhibitor, and 2 μg/ml lima trypsin inhibitor). Cells were harvested by scraping and then centrifuged for 5 min at 4 °C. For immunoblot analyses, 20 μg of cellular protein was resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies using protocols provided by the suppliers. Densitometric analyses of the Western blots were performed using a ChemiImager 4000 (Alpha-Innotech). When indicated, for the experiments showing densitometry of Western blots, the ordinate is in arbitrary units.

NOS Activity Assay—eNOS activity was quantified as the formation of L-[^{3}H] citrulline from L-[^{3}H] arginine in cultured BAEC, as described previously in detail (19, 29). Briefly, reactions were initiated by adding L-[^{3}H]arginine (10 μCi/ml) plus VEGF or S1P, as described below. Each treatment was performed in duplicate cultures, which were then each analyzed in duplicate. The flow-through fraction was analyzed by liquid scintillation counting, and NOS activity was quantitated based on L-[^{3}H]citrulline formation in the cells; the values were expressed as fmol of L-[^{3}H]citrulline produced/well/min.

Rac1 Activity Assay—Transfected BAEC in 100-mm dishes were stimulated with VEGF or S1P, and cells were then washed with ice-cold Tris-buffered saline and lysed in lysis buffer (25 mM HEPES, 150 mM NaCl, 1% Nonidet P-40, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 2 mM Na_3VO_4, 1 mM NaF). Pull-down of GTP-bound Rac was performed by incubating the cell lysates with glutathione *S*-transferase fusion protein corresponding to the p21-binding domain of p21-activated kinase-1 bound to glutathione-agarose (Upstate Biotechnology) for 1 h at 4 °C following the instructions provided by the suppliers. The beads were washed three times for 10 min each with lysis buffer, and the protein bound to the beads was eluted with 2× Laemmli buffer and analyzed for the amount of GTP-bound Rac by immunoblotting using a Rac monoclonal antibody.

Migration Assay—Cell migration was assayed using a Transwell cell culture chamber containing polycarbonate membrane inserts with an 8-μm pore (Corning Costar Corp.) coated with 0.2% gelatin (24). 48 h after transfection in 6-well plates, the cells were trypsinized, and 5×10^4 cells in 100 μl of Dulbecco's modified Eagle's medium, 0.4% fetal bovine serum were added to the upper Transwell chamber. The bottom chamber was filled with 600 μl of media, and the cells were allowed to adhere to the membrane at 37 °C for 1 h. VEGF (10 ng/ml), S1P (100 nM), or vehicle was added to the lower chamber, and the assembly was incubated at 37 °C for 3 h to allow cell migration. After incubation, the membranes were washed with phosphate-buffered saline, and the cells that did not migrate through the membrane were gently removed from the upper surface with a cotton swab. The membranes were then treated with trypsin to detach the migrated cells from the lower surface, and these cells were then counted with a hemocytometer.

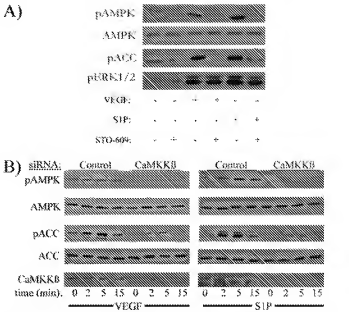

FIGURE 3. **Effects of the CaMKK inhibitor STO-609 or CaMKK siRNA on VEGF- and S1P-induced AMPK and ACC phosphorylation.** In *A*, BAEC lysates were prepared from cells pretreated with STO-609 at the indicated concentrations or its vehicle and then treated with VEGF (10 ng/ml) or S1P (100 nM) for 2 min. Cell lysates were resolved by SDS-PAGE and probed using antibodies directed against phospho-AMPK, AMPK, phospho-ACC, and phospho-ERK1/2. Shown are the representative data of three independent experiments. *B*, immunoblots of cell lysates prepared from BAEC that had been transfected 48 h earlier with control or CaMKKβ-specific siRNA. Cell lysates were resolved by SDS-PAGE and probed with the antibodies shown. An anti-CaMKKβ antibody was used to confirm siRNA-mediated CaMKKβ down-regulation. The experiment was repeated three times with equivalent results.

Tube Formation Assay—250 μl of Matrigel (BD Biosciences) was deposited into wells in a 24-well plate and allowed to solidify for 30 min at 37 °C. 48 h after siRNA transfection, BAEC were trypsinized, and 3×10^4 cells were added to each Matrigel-coated well. Cells were incubated on Matrigel for 9 h at 37 °C and imaged by phase-contrast microscopy (Nikon Eclipse TS100, ×5 objective). Four random fields of view (FOV)/well were examined and photographed by a blinded observer. For quantification purposes, a node was defined as an aggregation of cells from which three or more tubelike structures originated, and a tube referred to a continuous stretch of at least two cells containing no more than two nodes. For each FOV, ImageJ (National Institutes of Health) was used to measure the total tube length and the length per tube in units of pixels. Each experiment was repeated in nine wells.

Statistical Analysis—All experiments were performed at least three times. Mean values for individual experiments were expressed as means ± S.E. Statistical differences were assessed by ANOVA or *t* test when appropriate. A *p* value of less than 0.05 was considered significant.

RESULTS

VEGF- and S1P-mediated AMPK and ACC Phosphorylation—We first studied the effects of VEGF and S1P on the phosphorylation of AMPK and the AMPK substrate ACC in BAEC (Fig. 1). After the addition either of VEGF (10 ng/ml) or S1P (100 nM) to BAEC, AMPK phosphorylation increased within 1 min of agonist addition, reaching a maximum ~2.5-fold increase by 5

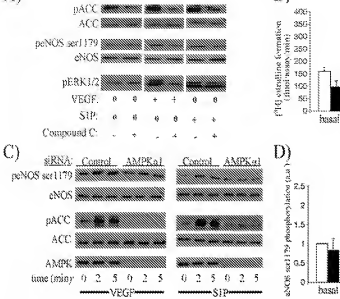

FIGURE 4. **Effects of pharmacologic inhibition or siRNA-mediated down-regulation of AMPK upon VEGF- and S1P-mediated eNOS activation.** *A*, BAEC lysates were prepared from cells that had been incubated with Compound C (20 μM) for 30 min or its vehicle and then treated with VEGF (10 ng/ml) or S1P (100 nM) for 5 min. Cell lysates were resolved by SDS-PAGE and probed using antibodies directed against phospho-ACC, ACC, phospho-eNOS, eNOS, and phospho-ERK1/2. The experiment shown is representative of three independent experiments that gave equivalent results. In *B*, transfected BAEC were assayed for eNOS enzyme activity by incubating the cells with a mixture of unlabeled L-arginine, [^{3}H]L-arginine, and VEGF (10 ng/ml) or S1P (100 nM) for 1 h, and cell extracts were processed to quantitate formation of [^{3}H]citrulline, as detailed under "Experimental Procedures." The addition of VEGF and S1P yielded a significant increase in eNOS activity in control siRNA-transfected cells ($p < 0.01$). *, $p < 0.05$ for AMPKα1 *versus* control siRNA-transfected cells (ANOVA). *C*, BAEC transfected with control or AMPKα1 siRNA were stimulated with VEGF (10 ng/ml) or S1P (100 nM) for the indicated times. Cells were lysed, and phosphorylation of eNOS and ACC were analyzed in the cell lysates in immunoblots probed with antibodies as shown. siRNA-mediated knockdown of AMPK was determined in immunoblots probed with AMPK antibody. *D*, densitometric analysis of pooled data, quantitating the relative eNOS phosphorylation in control and AMPKα1 siRNA-transfected cells under basal conditions and after treatment with VEGF or S1P for 5 min. Each data point represents the mean ± S.E. derived from four independent experiments; the addition of VEGF and S1P induced a significant increase in phosphorylation of eNOS in control siRNA-transfected cells ($p < 0.005$). *, $p < 0.05$ for AMPKα1 *versus* control siRNA-transfected cells (ANOVA).

between VEGF and S1P receptors in mediating intracellular endothelial responses. Indeed, it has been previously reported that S1P responses are mediated by VEGF receptor transactivation (31). We explored the possibility of S1P-mediated transactivation of the VEGF receptor 2 (VEGFR2) in promoting AMPK and ACC phosphorylation by using siRNA directed against VEGFR2. BAEC were transfected with duplex siRNAs specific for VEGFR2 (32), and the effects on siRNA-mediated VEGFR2 knockdown on VEGF- and S1P-induced AMPK and ACC phosphorylation were analyzed (Fig. 2*A*). VEGFR2 siRNA effectively abrogated AMPK and ACC phosphorylation in response to VEGF, but this siRNA did not affect phosphorylation in response to S1P. These findings indicate that VEGF, but not S1P, acts through the VEGFR2 to activate AMPK and ACC phosphorylation and argue against S1P-mediated VEGFR2 transactivation as an essential component of S1P-mediated AMPK activation.

We next used a series of pharmacological inhibitors to assess the role of tyrosine kinases and PI3K in VEGF- and S1P-mediated activation of AMPK and ACC. BAEC were stimulated with agonists following pretreatment with the broad spectrum tyrosine kinase inhibitor genistein, with the Src tyrosine kinase inhibitor PP2, with the PI3K inhibitor wortmannin, with the calcineurin inhibitor cyclosporin, or with the vehicle as a control (Fig. 2, *B–D*). We found that genistein and PP2 blocked VEGF-induced AMPK and ACC phosphorylation but did not affect S1P-induced AMPK and ACC phosphorylation. In contrast, pretreatment of BAEC with the PI3K inhibitor wortmannin did not affect AMPK or ACC phosphorylation in response to VEGF (data not shown), but wortmannin pretreatment significantly *increased* phosphorylation of both proteins in response to S1P (42.4 ± 18% increase compared with vehicle-pretreated, $n = 3$, $p < 0.05$) (Fig. 2, *C* and *D*). We found that the p38 inhibitor SB203580 had no effect in either VEGF- or S1P-induced AMPK or ACC phosphorylation, with positive controls affirming that p38 phosphorylation was partially blocked under these conditions (Fig. 2*C*) (33).

Effects of CaMKK Inhibition on VEGF- and S1P-mediated AMPK Phosphorylation Pathways—Both VEGF and S1P increase intracellular calcium in endothelial cells (20), and we sought to characterize a possible calcium-dependent upstream kinase responsible for both VEGF- and S1P-induced AMPK phosphorylation. BAEC incubated with the specific CaMKK

min, with a gradual return to basal levels at ~30 min following VEGF or S1P addition (Fig. 1, *A* and *B*). Immunoblots probed with a phospho-specific ACC antibody revealed a similar time course for ACC phosphorylation, reaching a peak ~3-fold increase in response to both agonists that gradually returned to base line after ~30 min following the addition of VEGF or S1P. We next analyzed the dose response to VEGF and S1P for both AMPK and ACC phosphorylation. Fig. 1*C* shows immunoblots of BAEC lysates from cells treated for 5 min with increasing concentrations of VEGF or S1P and probed with antibodies directed against phospho-AMPK and phospho-ACC; total AMPK and ACC levels serve as the control in this immunoblot analysis. The dose response for VEGF-induced AMPK and ACC phosphorylation demonstrated an EC_{50} of ~1 ng/ml, and S1P-induced phosphorylation of both targets showed an EC_{50} of ~20 nM; these values fall within the physiological range seen for many other endothelial responses for VEGF and S1P (20). Together, these data indicate that both VEGF and S1P induce the reversible receptor-mediated phosphorylation of AMPK and its substrate ACC in endothelial cells.

siRNA-mediated Down-regulation of VEGFR2 and Inhibition of Tyrosine Kinases and PI3K in VEGF- and S1P-mediated AMPK and ACC Phosphorylation—Previous work from our laboratory (30) and others (31) have observed cross-talk

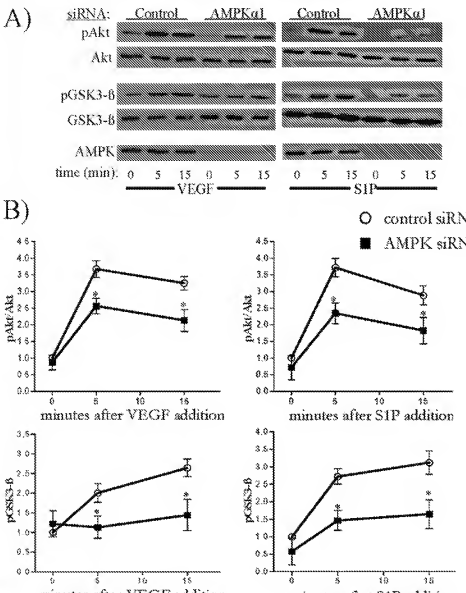

FIGURE 5. **Effects of siRNA-mediated down-regulation of AMPK on VEGF- and S1P-mediated Akt phosphorylation.** In *A*, BAEC transfected with control or AMPKα1 siRNA were treated with VEGF (10 ng/m) or S1P (100 nM) for the indicated times. Cell lysates were resolved on SDS-PAGE and analyzed in immunoblots probed with specific antibodies directed against phospho-Akt, Akt, phospho-GSK3-β, GSK3-β, and AMPK. Shown are the results of a representative experiment that was repeated four times with equivalent results. *B*, pooled data plotting the ratio between the signal intensity of phospho-Akt and total Akt or phospho-GSK3-β and total GSK3-β as determined by densitometry. Each point in the graph represents the mean ± S.E. of four independent experiments. *, $p < 0.05$ *versus* control siRNA-transfected cells (using ANOVA).

inhibitor STO-609 (34, 35) did not demonstrate agonist-stimulated AMPK and ACC phosphorylation in response to either VEGF or S1P (Fig. 3*A*). We next designed and validated siRNA targeting the bovine β isoform of CaMKK; transfection with this siRNA severely impaired both VEGF- and S1P-induced AMPK and ACC phosphorylation (Fig. 3*B*). These findings suggest that CaMKK may link VEGF- and S1P-activated pathways upstream of AMPK phosphorylation.

siRNA-mediated Down-regulation of AMPK Impairs Agonist-mediated eNOS and Akt Activation—We next used siRNA approaches to explore the role of AMPK in VEGF- and S1P-mediated eNOS and Akt activation in BAEC. Pretreatment of endothelial cells with the potent and selective AMPK inhibitor Compound C (36) inhibited ACC phosphorylation in response to both agonists and also attenuated VEGF- and S1P-induced eNOS phosphorylation at its activating serine 1179 site without affecting ERK1/2 phosphorylation (Fig. 4*A*). We then designed and validated a duplex siRNA construct targeting the catalytic α-subunit of AMPK; immunoblotting for total AMPK and for phospho-ACC confirmed ~90% down-regulation of AMPK expression (Fig. 4*C*). As shown in Fig. 4*B*, down-regulation of AMPK attenuated both VEGF- and S1P-mediated enhancement in eNOS activity, as determined by the intracellular formation of L-[^{3}H]citrulline from L-[^{3}H]arginine (49 ± 14% decrease in VEGF-induced eNOS activity for AMPK siRNA compared with control siRNA; 43 ± 19% decrease for S1P-induced eNOS activity; $n = 4$ independent experiments performed in duplicate, $p < 0.05$ for both). siRNA-mediated AMPK knockdown significantly attenuated both VEGF- and S1P-induced eNOS Ser^{1179} phosphorylation compared with control siRNA-treated cells (Fig. 4, *C* and *D*).

Relative Roles of AMPK and Akt in Agonist-mediated eNOS Activation—After identifying AMPK as a critical component of the pathway involved in VEGF- and S1P-induced eNOS activity, we analyzed the relationship between AMPK and another important eNOS kinase, Akt, to determine how these two kinases may interact in regulating eNOS. As shown in Fig. 5, AMPK siRNA significantly impaired the VEGF- and S1P-mediated phosphorylation of Akt at its serine 473 site and also blocked phosphorylation of the kinase Akt substrate glycogen synthase kinase-3-β (GSK3-β), suggesting that AMPK lies upstream of Akt in this pathway. We next designed and validated duplex siRNA-targeting constructs specific for Akt1, the predominant Akt isoform in endothelial cells (37, 38); immunoblotting for total Akt1 confirmed ~80–90% knockdown of Akt1, and GSK3-β phosphorylation was efficiently blocked following siRNA-mediated Akt1 knockdown (Fig. 6, *B* and *C*). BAEC transfected with Akt1 siRNA showed a significant decrease in eNOS activity; as shown in Fig. 6*A*, there was a 48 ± 5% decrease in eNOS activity in Akt1 siRNA- *versus* control

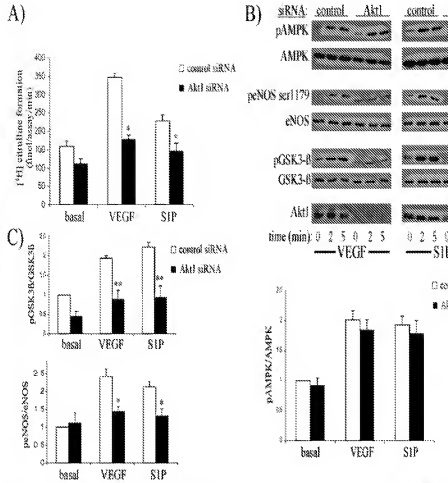

FIGURE 6. **Effects of siRNA-mediated down-regulation of Akt1 on VEGF- and S1P-mediated eNOS activation and AMPK phosphorylation.** *A*, BAEC transfected with control or Akt1 siRNA were assayed for eNOS enzyme activity as described above. The addition of VEGF and S1P induced a significant increase in eNOS activity in control siRNA-transfected cells ($p < 0.01$). *, $p < 0.05$ for Akt1 *versus* control siRNA-transfected cells. *B*, BAEC transfected with control or Akt1 siRNA were stimulated with VEGF or S1P for the indicated times. Cells were lysed, and phosphorylation of AMPK, eNOS, and GSK3-β were analyzed in the cell lysates in immunoblots probed with phospho-specific antibodies as shown. *C*, densitometric analysis of pooled data, presenting the ratio between phosphorylated and total eNOS, phosphorylated and total AMPK, and phosphorylated and total GSK3-β in control and Akt1 siRNA-transfected cells, both under basal conditions and after treatment with VEGF and S1P for 5 min. Each data point represents the mean ± S.E. derived from four independent experiments; the addition of VEGF and S1P induced a significant increase in phosphorylation of eNOS, GSK3-β, and AMPK in control siRNA-transfected cells ($p < 0.01$). *, $p < 0.05$; **, $p < 0.01$ for Akt1 *versus* control siRNA-transfected cells.

siRNA-transfected cells in response to VEGF and a 37 ± 11% decrease in eNOS activity in response to S1P ($n = 3, p < 0.05$ for both). There was also an accompanying decrease in eNOS Ser^{1179} phosphorylation in Akt1 siRNA-transfected cells (Fig. 6, *B* and *C*; for VEGF, there was a 40 ± 13% reduction in eNOS Ser^{1179} phosphorylation in Akt1 siRNA- *versus* control siRNA-transfected cells; for S1P, there was a 39 ± 23% decrease in agonist-mediated eNOS Ser^{1179} phosphorylation; $n = 4$, $p < 0.05$ for both). Importantly, siRNA-mediated Akt1 knockdown did *not* affect either VEGF- or S1P-induced AMPK phosphorylation (Fig. 6, *B* and *C*). These findings suggest that AMPK lies upstream of Akt in VEGF- and S1P-mediated eNOS activation and indicate that Akt might act as an effector of AMPK in this response.

Role of Rac1 in Agonist-modulated, AMPK-mediated eNOS Activation—The small GTPase Rac1, which functions as a molecular switch by cycling between an inactive GDP-bound form and an active GTP-bound form, is a well characterized regulator of the actin cytoskeleton, but its roles in the Akt/PI3K pathway remain controversial (27, 39, 40). We have previously shown that Rac1 is a key determinant of Akt phosphorylation in response to S1P (27). In the present studies, we explored the role of Rac1 in modulation of AMPK responses involved in eNOS activation (Fig. 7). BAEC were transfected with either control or AMPK siRNA, and agonist-induced activation of Rac1 was measured in the cell lysates by pull-down of activated GTP-Rac1. We observed that siRNA-mediated AMPK knockdown impaired Rac1 activity in response to both S1P (53 ± 17% decrease *versus* control siRNA; $n = 3$, $p < 0.02$) and VEGF (48 ± 21% decrease; $n = 3$, $p < 0.05$), indicating that siRNA-mediated AMPK knockdown attenuates agonist-induced Rac1 activation. We next transfected BAEC with Rac1 siRNA (27) and found that siRNA-mediated Rac1 knockdown significantly impaired both VEGF- and S1P-stimulated phosphorylation of the AMPK substrate ACC and of eNOS, but *without* affecting phosphorylation of AMPK (Fig. 8).

Caveolin-1 Regulates AMPK Phosphorylation—After identifying a role for Rac1 as a signaling molecule downstream of AMPK, we next explored the relationship between AMPK and the scaffolding/regulatory protein caveolin-1. We previously reported that siRNA-mediated caveolin-1 knockdown significantly enhances both Rac1 activity and Akt phosphorylation (24). To explore the possible role for caveolin-1 in AMPK responses, we transfected BAEC with caveolin-1 siRNA (24) and analyzed the cell lysates in immunoblots probed with phospho-AMPK- and phospho-GSK3-β-specific antibodies (Fig. 9). As we found previously, caveolin-1 knockdown significantly increased GSK3-β phosphorylation. Importantly, siRNA-mediated caveolin-1 knockdown also reproducibly potentiated basal AMPK phosphorylation (2.5 ± 0.2-fold increase in basal phosphorylation compared with control siRNA-treated cells, $p < 0.01$) and also enhanced the agonist-mediated response to VEGF (40 ± 10% increase in AMPK phosphorylation, $p < 0.01$) as well as S1P (44 ± 11% increase, $p < 0.01$), as shown in Fig. 9, *B* and *C*. This finding suggests that caveolin-1 acts as a negative regulator of agonist-mediated AMPK phosphorylation in endothelial cells.

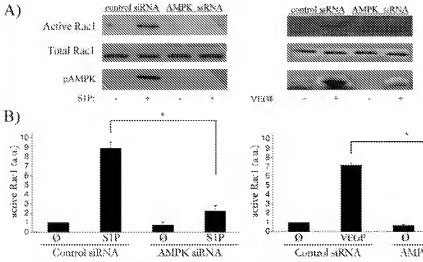

FIGURE 7. **Effects of AMPK siRNA on agonist-enhanced Rac1 activity.** *A*, BAEC were transfected with control or AMPKα1 siRNA and subsequently treated with S1P (100 nM) for 5 min or with VEGF (10 ng/ml) for 1 min; Rac1 activity in the cell lysates was measured by pull-down of GTP-Rac1 using the glutathione *S*-transferase-p21-binding domain of p21-activated kinase-1. Active and total Rac1 were detected in immunoblots using an anti-Rac1-specific antibody. Phospho-AMPK in the cell lysates was detected in immunoblots as shown. *B*, densitometric analysis of pooled data showing the ratio between active and total Rac1 after treatment with S1P and VEGF. Each data point represents the mean ± S.E. derived from three independent experiments; the addition of S1P and VEGF induced a significant increase in Rac1 activity ($p < 0.01$). **, $p < 0.02$ versus* S1P- or VEGF-treated control siRNA-transfected cells (*t* test).

Role of AMPK Pathway in VEGF- and S1P-mediated Endothelial Cell Migration—We have previously shown that Rac1 activity is critical for endothelial migration in response to S1P (24, 27), and we hypothesized that AMPK modulates both VEGF- and S1P-induced endothelial cell migration through the Rac1-Akt-eNOS pathway described above. Fig. 10 shows the results of a cell migration assay using a Transwell cell culture chamber containing BAEC transfected with control siRNA or with siRNA constructs targeting caveolin-1, AMPK, Akt1, or eNOS, as shown. Transfection of BAEC with these siRNA-targeting constructs led to significant and specific knockdown of their cognate proteins (Fig. 10*A*). As shown in Fig. 10*B*, agonist-enhanced endothelial cell migration was significantly reduced in cells transfected with the AMPK siRNA (43 ± 10% decrease in VEGF-stimulated cell migration relative to control siRNA-transfected cells; 46 ± 14% decrease for S1P-induced migration relative to control siRNA-transfected cells; $n = 3$, $p < 0.05$). Agonist-induced cell migration was also attenuated by Rac1 siRNA (42 ± 8% attenuation of the response to VEGF and 39 ± 7% inhibition for S1P; $n = 3$, $p < 0.05$ for both). There was a similar attenuation of the migration response to VEGF (31 ± 13% decrease) and S1P (36 ± 17%) following transfection of Akt1 siRNA ($n = 3$, $p < 0.05$ for both agonists). Finally, eNOS siRNA transfection led to a marked reduction in migration responses to VEGF (44 ± 11%) as well as to S1P (48 ± 14%) ($n = 3$, $p < 0.004$ for both VEGF and S1P responses following eNOS siRNA transfection compared with control siRNA transfection, analyzed by ANOVA).

AMPK Mediates Endothelial Tube Formation via Rac1 and eNOS Signaling—After identifying a role for AMPK and several of its signaling partners in endothelial cell migration, we next explored the consequences of knockdown of AMPK and AMPK target proteins in the endothelial cell tube formation assay, an extensively validated *in vitro* model for angiogenesis (41, 42). siRNA-transfected BAEC were plated on Matrigel (41, 42) and allowed to form capillary-like tubes for 9 h; the tubes were then photographed and analyzed using novel quantification parameters. Compared with cells transfected with control siRNA, BAEC transfected with siRNA constructs targeting AMPK, Rac1, or eNOS siRNA, but not caveolin-1, exhibited a significantly reduction in tube formation. As shown in Fig. 11*B*, there was a 59 ± 6% reduction in average total tube length per field of view in cells transfected with AMPK siRNA, a 37 ± 7% reduction in cells transfected with Rac1 siRNA, and a 46 ± 4% reduction in cells transfected with eNOS siRNA, compared with cells transfected with control siRNA ($n = 9$ wells each, $p < 0.02$ for all conditions). We also measured the length of individual tubes and then performed population analyses for each experimental condition. In analyzing this quantification parameter, we found that cells that had been transfected with AMPK, Rac1, or eNOS siRNA had shorter individual tubes (Fig. 11*C*) as well as a reduction in the aggregate length of endothelial tubes.

DISCUSSION

We have pursued pharmacological approaches along with siRNA-mediated knockdown methods to study the molecular mechanisms involved in VEGF and S1P signaling to AMPK. In these studies, we have explored the upstream modulators of agonist-mediated AMPK phosphorylation and have identified a novel AMPK → Rac1 → Akt pathway that functions as a critical determinant of eNOS activity, as well as endothelial migration and tube formation, in the vascular endothelium (see model in Fig. 12).

Although traditionally thought to be regulated by the cellular energy state alone, AMPK is now known to be activated by calcium-dependent signaling pathways (for a review, see Ref. 43). In endothelial cells, AMPK is known to phosphorylate the Ser^{1179} site on eNOS, thereby increasing the V_{max} of the enzyme and its sensitivity to calcium and calmodulin (8). Both the polypeptide growth factor VEGF and the lipid mediator S1P are known to activate eNOS in endothelial cells in part through mobilization of calcium (20), but previous studies have not clearly demonstrated AMPK-dependent pathways in these agonist-mediated responses. Although a recent report has implicated AMPK in VEGF-stimulated endothelial NO production (18), the mechanisms linking VEGF stimulation with AMPK and eNOS activation were not explored. Furthermore, it has not been shown whether S1P can also regulate AMPK upstream of eNOS activation. In the present studies, we conclusively identify AMPK as a mediator of VEGF- and S1P-mediated eNOS activation, in the absence of any other known AMPK

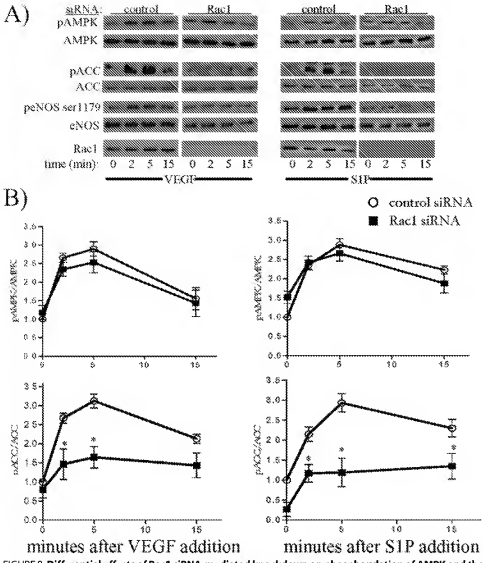

FIGURE 8. **Differential effects of Rac1 siRNA-mediated knockdown on phosphorylation of AMPK and the AMPK substrates ACC and eNOS.** *A* shows the results of immunoblots analyzed in BAEC lysates prepared from cells transfected with control or Rac1 siRNA and treated with VEGF or S1P for the indicated times. Cell lysates were resolved by SDS-PAGE and probed using antibodies directed against phospho-AMPK, AMPK, phospho-ACC, ACC, phospho-eNOS, eNOS, and Rac1. Shown are the representative data of three independent experiments. *B*, the results of densitometric analyses from pooled data, plotting the ratios of phosphorylated ACC and AMPK to total ACC and AMPK, respectively, relative to the signals present in the unstimulated control siRNA-transfected cells. Each data point represents the mean ± S.E. derived from three independent experiments. *, $p < 0.05$ *versus* control siRNA-transfected cells (ANOVA).

regulators. In BAEC, VEGF and S1P were found to reversibly phosphorylate AMPK at its activation site, Thr^{172}, along with phosphorylation of the AMPK substrate ACC (Fig. 1). We found that this phosphorylation occurred at EC_{50} values (~1 ng/ml for VEGF and ~10–20 nM for S1P) characteristic of those observed with other receptor-mediated effects of these agonists (reviewed in Ref. 20). In contrast to our findings, Zou *et al.* (44) found that VEGF did not affect AMPK activity either administered alone or in combination with the AMPK activator peroxynitrite, and Nagata *et al.* (12) did not observe a significant role for AMPK in VEGF-mediate angiogenesis under normoxic conditions. We note that in these studies, cells were stimulated with VEGF at concentrations of 50 ng/ml (12, 44) for as long as 6 h (12), a dose and time point at which we also did not observe the stimulatory effects of VEGF upon AMPK.

In contrast to the VEGF receptor tyrosine kinase, S1P is known to facilitate its endothelial cell functions by interacting with G protein-coupled S1P receptors (45, 46). Despite these differential modes of receptor activation, we have previously found that the Src tyrosine kinase inhibitor PP2 blocks S1P-mediated Rac1 activation and Akt phosphorylation (27), thereby implicating tyrosine kinases in S1P-mediated signal transduction. In the present studies, however, we observed that PP2, as well as the broad spectrum tyrosine kinase inhibitor genistein, diminished VEGF-mediated AMPK and ACC phosphorylation but did not affect these responses mediated by S1P (Fig. 2*B*). Taken together with our previous work, it appears that the role of Src in endothelial cells is agonist-dependent, since Src appears to lie upstream of AMPK when cells are stimulated by VEGF but downstream of AMPK when cells are stimulated by S1P. We found further that VEGFR2 siRNA impaired VEGF- but not S1P-mediated AMPK and ACC phosphorylation (Fig. 2*A*), thereby indicating that VEGFR2 does not play a role in the S1P-stimulated AMPK pathway. In addition to the VEGFR2 and tyrosine kinases, our work identifies PI3K as another important locus of differential regulation of AMPK by VEGF *versus* S1P; although the PI3K inhibitor wortmannin did not affect VEGF-induced AMPK and ACC phosphorylation at doses sufficient for inhibition of Akt activation, wortmannin substantially increased phosphorylation of both targets in response to S1P (Fig. 2*C*). These results suggest divergent receptor-modulated PI3K-independent pathways upstream of AMPK (47) and lead us to speculate that although both agonists activate AMPK in a PI3K-independent manner, S1P, but not VEGF, might also activate a competitive PI3K-dependent pathway whose inhibition results in hyperphosphorylation of AMPK. Although VEGF and S1P activate differential pathways upstream of AMPK, our studies implicate CaMKKβ as a common upstream modulator of AMPK phosphorylation. Indeed, both the CaMKK inhibitor STO-609 and CaMKKβ siRNA effectively abrogated both VEGF- and S1P-mediated AMPK and ACC phosphorylation (Fig. 3). However, the complexities of calcium/calmodulin-dependent responses in AMPK signaling and the fact that eNOS

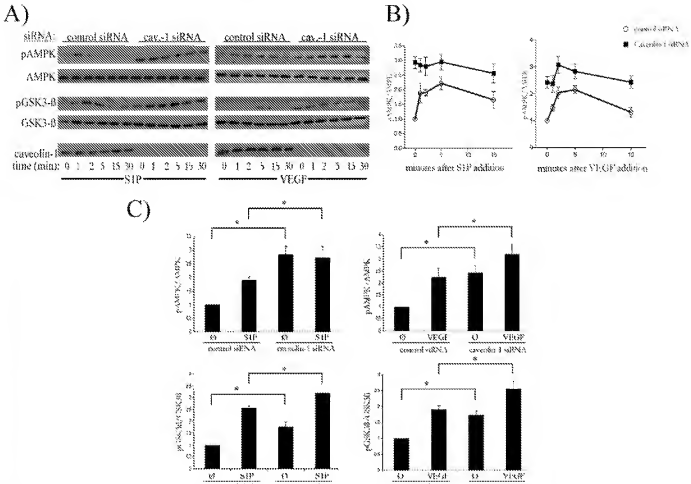

FIGURE 9. **siRNA-mediated caveolin-1 knockdown enhances AMPK phosphorylation.** *A*, BAEC were transfected with control or caveolin-1 siRNA; 48 h after transfection, cells were treated with VEGF (10 ng/ml) or S1P (100 nM) for the indicated times. Cell lysates were resolved by SDS-PAGE and analyzed in immunoblots probed with antibodies specific to the proteins or phosphoproteins as shown. Caveolin-1 expression was analyzed in immunoblots with anti-caveolin-1 antibody. Shown are the representative results of an experiment that was repeated three times with equivalent results. *B*, pooled data from time course experiments in which the relative intensity of each data point in VEGF- and S1P-treated cells was analyzed by densitometry. Each point in the graph represents the mean ± S.E. of three independent experiments. *C*, the results of densitometric analyses showing the ratios of phospho-AMPK to total AMPK and phospho-GSK3-β to total GSK3-β in caveolin-1 siRNA or control siRNA-transfected cells in basal conditions and after treatment with VEGF or S1P for 2 min. Each data point represents the mean ± S.E. derived from three independent experiments. The addition of either VEGF or S1P induced a significant increase in phosphorylation of AMPK ($p < 0.005$). *, $p < 0.001$ for caveolin-1 *versus* control siRNA-transfected cells (ANOVA).

itself is modulated by calcium/calmodulin together hamper our ability to discern the detailed mechanisms whereby CaMKKβ regulates receptor-dependent signaling to eNOS.

The question of whether AMPK is a critical component of the receptor-modulated signaling pathways leading to eNOS activation appears to vary depending on the particular agonist. For example, both 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside-induced (17) and receptor-mediated activation of eNOS by histamine (47), adiponectin (12), or estradiol (48) depend at least partially upon AMPK. By contrast, AMPK reportedly is not involved in other receptor-mediated cell signaling pathways that result in eNOS activation or nitric oxide release, such as those stimulated by insulin (11), thrombin (36), or bradykinin (49). In these studies, we observed that either pharmacologic inhibition or siRNA-mediated down-regulation of the catalytic subunit of AMPK impaired both eNOS activity and eNOS phosphorylation at its activating Ser^{1179} site in response to either VEGF or S1P (Fig. 4). These results establish that AMPK is a critical component of the eNOS-activating pathways modulated by both VEGF and S1P.

The critical role of AMPK in VEGF- and S1P-induced eNOS activity and phosphorylation led us to explore the role of another eNOS kinase, Akt. There is a complex and incompletely understood relationship between AMPK and Akt in both cardiac myocytes and endothelial cells, with several recent reports highlighting the importance of AMPK-Akt cross-talk in modulating agonist-mediated AMPK activities (16, 17, 50, 51). Horman *et al.* (50) recently proposed a mechanism in rat hearts whereby insulin treatment decreased AMPK Thr^{172} phosphorylation in an Akt-dependent manner, thereby identifying Akt as a negative regulator of AMPK activity in response to insulin. Similarly, Soltys *et al.* (51) found that activation of Akt1 prevents activation of AMPK by LKB1 in isolated cardiac myocytes by phosphorylating the regulatory $Ser^{485/491}$ site on AMPK. By contrast, our studies in BAEC with Akt1 siRNA (Fig. 6) argue strongly against an upstream role for Akt in VEGF- and S1P-

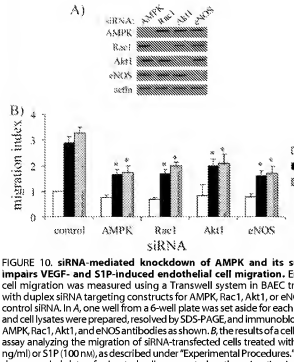

FIGURE 10. **siRNA-mediated knockdown of AMPK and its substrates impairs VEGF- and S1P-induced endothelial cell migration.** Endothelial cell migration was measured using a Transwell system in BAEC transfected with duplex siRNA targeting constructs for AMPK, Rac1, Akt1, or eNOS or with control siRNA. In *A*, one well from a 6-well plate was set aside for each condition, and cell lysates were prepared, resolved by SDS-PAGE, and immunoblotted using AMPK, Rac1, Akt1, and eNOS antibodies as shown. *B*, the results of a cell migration assay analyzing the migration of siRNA-transfected cells treated with VEGF (10 ng/ml) or S1P (100 nM), as described under "Experimental Procedures." The panel shows pooled data of migrated cells, presented as the *migration index*, which represents the number of migratory cells following siRNA transfection/number of migratory cells determined under basal conditions in control siRNA-transfected BAEC. For all cells, the addition of VEGF or S1P induced a significant increase in endothelial cell migration ($p < 0.05$). *, $p < 0.05$ *versus* corresponding treatment in control siRNA-transfected cells (using ANOVA).

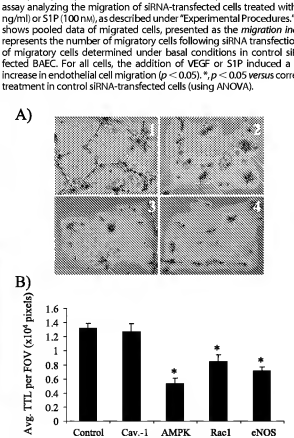

FIGURE 11. **AMPK-dependent endothelial tube formation.** BAEC were transfected with control, caveolin-1, AMPK, Rac1, or eNOS siRNA; 48 h after transfection, the cells were trypsinized and resuspended in fetal bovine serum-supplemented medium, and 3×10^4 cells were plated into each well of a 24-well plate coated with Matrigel. The tubes were photographed and analyzed by a blinded observer 9 h after plating. *A*, representative images of endothelial tube formation by BAEC transfected with control siRNA (*1*), AMPK siRNA (*2*), Rac1 siRNA (*3*), and eNOS siRNA (*4*). *B*, the average total tube length (*TTL*) per FOV is shown with S.E. derived from nine independent experiments. *, a statistically significant difference ($p < 0.02$) compared with the total tube length per FOV of BAEC transfected with control siRNA. *C*, individual tube length (in number of pixels) is grouped into nine categories: 1 (0–49 pixels), 2 (50–99 pixels), 3 (100–149 pixels), 4 (150–199 pixels), 5 (200–249 pixels), 6 (250–299 pixels), 7 (300–349 pixels), and 8 (350–399 pixels).

mediated AMPK activation, since Akt1 knockdown impaired eNOS activity and Ser^{1179} phosphorylation but did not affect AMPK phosphorylation in response to either agonist. It may be that the Akt-AMPK interaction depends importantly upon the cell type and the specific receptor-mediated pathway at play. Whether Akt lies downstream and not just parallel to AMPK is also controversial and appears to be cell type-specific and agonist-dependent. For example, Morrow *et al.* (17) found that expression of dominant negative AMPK had no effect on 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside-stimulated Akt phosphorylation in human aortic endothelial cells. By contrast, Ouchi *et al.* (16) showed that inhibition of AMPK signaling with dominant negative constructs suppressed adiponectin-induced Akt phosphorylation, suggesting that Akt lies downstream of AMPK in adiponectin-activated endothelial cell pathways. Our data using AMPK siRNA are consistent with these latter observations, in which our studies clearly establish that AMPK functions upstream of Akt in VEGF- and S1P-mediated eNOS activation (Fig. 5) and thereby implicate an AMPK → Akt → eNOS pathway in response to these agonists. It is worth noting that siRNA-mediated knockdown of Akt1 did not lead to AMPK or ACC hyperphosphorylation, in contrast to the hyperphosphorylation effect seen with the PI3K inhibitor wortmannin. We speculate that this difference between the effects of wortmannin *versus* Akt1 siRNA may reflect Akt-independent signaling downstream of PI3K, perhaps involving the MAPK pathway, as has been proposed in a number of recent reports (52, 53). Further studies will be necessary to determine whether AMPK can directly activate eNOS in a pathway that does not involve Akt.

The present studies have also implicated Rac1 in a novel role downstream of the activation of AMPK. Rac1 has been previously implicated in a broad range of both VEGF- and S1P- mediated cellular responses in vascular endothelial cells, including nitric oxide release, migration, and angiogenesis (54). We have recently reported that S1P-enhanced Rac1 activation is required for the activation of Akt and eNOS (27). Our identification of an AMPK-Akt-eNOS axis in VEGF- and S1P-treated endothelial cells therefore led us to hypothesize that Rac1 might play a role in the AMPK-mediated modulation of Akt and eNOS. We discovered that siRNA-mediated AMPK knockdown significantly impaired both VEGF- and S1P-induced Rac1 activation (Fig. 7), suggesting that AMPK is required for receptor-mediated Rac1 activation. By contrast,

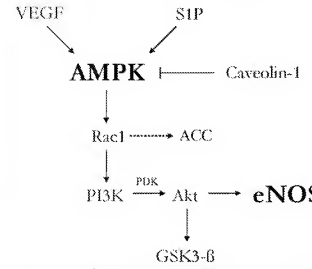

FIGURE 12. **Scheme for VEGF- and S1P-mediated regulation of AMPK in BAEC.** In this model, VEGF and S1P differentially phosphorylate AMPK, which can then activate Rac1. Rac1 activation, in turn, is required for the activation of PI3K, which then, via phosphoinositide-dependent kinases, activates Akt. Akt then phosphorylates GSK3-β and eNOS. Caveolin-1 acts as a negative regulator of AMPK. Rac1 also modulates AMPK-mediated ACC phosphorylation. See "Discussion" for details.

siRNA-mediated Rac1 knockdown impaired VEGF- and S1P-induced eNOS and ACC phosphorylation but had no effect on agonist-induced AMPK phosphorylation (Fig. 8), thereby identifying Rac1 as a critical regulator of downstream AMPK targets but not of AMPK itself. Taken together with our recent finding that Rac1 knockdown also attenuates phosphorylation of Akt and its substrate GSK3-β (27), the present studies suggest that Rac1 might act as an AMPK effector in modulating AMPK-dependent regulation of both ACC and the agonist-mediated Akt/eNOS pathway in endothelial cells. To our knowledge, this is the first report of a link between AMPK and Rac1. Indeed, it is tempting to speculate that AMPK-mediated regulation of Rac1 might play a role in cellular defense against oxidative stress, since both proteins have been implicated in endothelial cell redox signaling and cardioprotection (55, 56).

In addition to AMPK, caveolin-1 represents another important regulator of Rac1 activity. We have previously reported that siRNA-mediated knockdown of caveolin-1 significantly enhances both Rac1 activity and Akt phosphorylation, suggesting a mechanism whereby caveolin-1 negatively regulates Rac1, which in turn modulates the PI3K/Akt/eNOS pathway in endothelial cells (24). In the present studies, we were intrigued to find that siRNA-mediated caveolin-1 knockdown enhanced AMPK phosphorylation both in the basal state and in response to VEGF and S1P (Fig. 9), thereby suggesting that caveolin-1 also negatively regulates AMPK activity. Interestingly, the increase in AMPK phosphorylation seen with caveolin-1 knockdown appeared to effectively "uncouple" VEGF- and S1P-stimulated AMPK phosphorylation, suggesting that abrogation of the inhibitory effect of caveolin-1 on AMPK phosphorylation with caveolin-1 siRNA might eclipse the otherwise stimulatory effect of these receptor-dependent AMPK agonists. We note that other kinases, such as protein kinase A (57), as well as phosphoprotein phosphatases (58) have been implicated in ACC regulation. It is plausible that these other mechanisms may play a role in enhancing ACC activity when AMPK phosphorylation is stimulated following caveolin-1 knockdown. Importantly, we also did not observe statistically significant differences in eNOS phosphorylation or activity in caveolin-1 siRNA- *versus* control siRNA-transfected cells (data not shown), consistent with our previous work (24). We note that although eNOS is a target of AMPK, eNOS activation depends upon many other signaling proteins, including protein kinase A (59) and the protein phosphatase PP2A (60), whose role in eNOS regulation may counterbalance the anticipated increase in eNOS phosphorylation after caveolin-1 knockdown. Caveolin-1 may even directly interact with these other eNOS regulators in a way that would attenuate an increase in eNOS phosphorylation. For example, Li *et al.* (61) have reported that overexpression of caveolin-1 inhibits protein phosphatases PP1 and PP2A in prostate cancer cells. Possibly, siRNA-mediated caveolin-1 knockdown might remove this inhibitory effect on these phosphatases and thereby enhance the dephosphorylation of eNOS, thus attenuating the enhancement in eNOS phosphorylation consequent to activation of AMPK following caveolin-1 knockdown. Both the complex upstream regulation of eNOS and the pleiotropic effects of caveolin-1 in endothelial cells may explain why caveolin-1 knockdown does not significantly affect eNOS phosphorylation at Ser^{1179} despite a robust enhancement of AMPK phosphorylation. Further studies of agonist-modulated AMPK pathways may help to further define the inhibitory interaction between caveolin-1 and AMPK.

Our studies also contribute insights into the molecular mechanisms involved in eNOS-dependent endothelial migration and tube formation, two key endothelial functional responses involved in many processes, including wound healing, vascular development, and angiogenesis (26, 41). Both VEGF (62) and S1P (63) are known to stimulate endothelial cell migration, mediated in part through eNOS signaling; our study provides new information implicating AMPK in the modulation of eNOS activation associated with agonist-induced cell migration responses (Fig. 10). Extending our previous work that implicated Rac1 in endothelial migration (24), these studies have also identified a context for Rac1 as an AMPK mediator in regulating endothelial migration. We have also exploited siRNA-based approaches to explore the roles of AMPK and its modulators in endothelial tube formation upon the basement membrane-like matrix Matrigel. The Matrigel tube formation assay is an extensively validated method that permits the quantitative analysis of the propensity for cultured endothelial cells to form capillary-like structures, providing a useful model for angiogenesis (41, 64). Using rigorous quantification methods, we found that BAEC transfected with AMPK, Rac1, or eNOS siRNA formed less extensive tubular networks as well as shorter individual tubes (Fig. 11), thereby indicating that eNOS is required for adequate endothelial tube formation in an AMPK- and Rac1-dependent manner.

With the advent of studies implicating alternative, AMP-independent pathways of AMPK activation (43), it now appears that the AMPK system functions to respond not only to cell autonomous signs of stress such as AMP but also to circulating factors that act as regulators of cellular function in both physiologic and pathophysiologic states. As key determinants of

many cellular processes in endothelial cells, VEGF and S1P represent intriguing new stimuli for AMPK activation. These studies have demonstrated that the potent eNOS agonists VEGF and S1P differentially phosphorylate and thereby activate AMPK in vascular endothelial cells and establish a central role for AMPK in VEGF- and S1P-mediated eNOS activity. We identify for the first time an AMPK → Rac1 → Akt pathway that functions as a critical determinant of eNOS activity and endothelial migration and tube formation in endothelial cells. These studies have provided new insights into the molecular mechanisms linking extracellular signals with AMPK in the modulation of eNOS activation in the vascular endothelium.

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Activation and signaling by the AMP-activated protein kinase in endothelial cells.

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The AMP-activated protein kinase (AMPK) was initially identified as the kinase that phosphorylates the 3-hydroxy 3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme for cholesterol biosynthesis. As the name suggests, the AMPK is activated by increased intracellular concentrations of AMP, and is generally described as a "metabolite-sensing kinase" and when activated initiates steps to conserve cellular energy. Although there is a strong link between the activity of the AMPK and metabolic control in muscle cells, the activity of the AMPK in endothelial cells can be regulated by stimuli that affect cellular ATP levels, such as hypoxia as well as by fluid shear stress, Ca(2+)-elevating agonists, and hormones such as adiponectin. To date the AMPK in endothelial cells has been implicated in the regulation of fatty acid oxidation, small G protein activity and nitric oxide production as well as inflammation and angiogenesis. Moreover, there is evidence indicating that the activation of the AMPK may help to prevent the vascular complications associated with the metabolic syndrome.

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Targeting the AMPK pathway for the treatment of Type 2 diabetes

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Abstract

Type 2 diabetes is one of the fastest growing public health problems worldwide resulting from both environmental and genetic factors. It is characterized by the abnormal glucose and lipid metabolism due in part to resistance to the actions of insulin in skeletal muscle, liver and fat. This may result from inadequate adaptation to environmental changes (e.g., imbalance between energy intake and energy expenditure). AMP-activated protein kinase (AMPK), a phylogenetically conserved serine/ threonine protein kinase, acts as an integrator of regulatory signals monitoring systemic and cellular energy status. The growing realization that AMPK regulates the coordination of anabolic (synthesis and storage of glucose and fatty acids) and catabolic (oxidation of glucose and fatty acids) metabolic processes represents an attractive therapeutic target for intervention in many conditions of disordered energy balance. Recent evidences that pharmacological activation of AMPK improves blood glucose homeostasis, lipid profile and blood pressure in insulin-resistant rodents, make this protein kinase a novel therapeutic target in the treatment of type 2 diabetes. Consistent with these results, physical exercise and two major classes of antidiabetic drugs (biguanides and thiazolidinediones) have recently been reported to activate AMPK. In the present review, we update these topics and discuss the concept of targeting AMPK pathway for the treatment of type 2 diabetes.

2 Introduction

The increased prevalence of obesity and type 2 diabetes, with the attendant increase in morbidity and mortality, pose a substantial therapeutic challenge. Type 2 diabetes is a complex polygenic disease with a strong genetic component, as indicated by the high prevalence in certain ethnic groups and by studies of identical twins. Nevertheless, the rapid increase in the prevalence of obesity-associated disease conditions, including type 2 diabetes, in worldwide populations suggests the contribution of environmental factors. A widely accepted explanation lays on the frequent consumption of processed foods with a high-calorie content and the reduction in physical exercise due to sedentary lifestyle in modern urban environment. Disruption of energy balance has led to an increased prevalence of these conditions (1). Type 2 diabetes is characterized by altered lipid and glucose metabolism (fasting or postprandial hyperglycemia, dyslipidemia) as a consequence of combined insulin resistance in skeletal muscle, liver and adipose tissue and relative defects of insulin secretion by β-cells that may arise due to an imbalance between energy intake and expenditure (2). Insulin resistance occurs when a normal dose of hormone is unable to elicit its metabolic responses. Insulin is the primary anabolic hormone that stimulates uptake and storage of fuel substrates, while inhibiting substrate production in peripheral tissues. It lowers blood glucose levels by facilitating glucose

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uptake, mainly into skeletal muscle and fat tissue, and by inhibiting endogenous glucose production in the liver. Peripheral insulin resistance is associated with lipid partitioning in specific compartments, i.e. muscle and liver, more than with obesity *per se*. Usually, after an asymptomatic period of insulin resistance, hyperglycemia appears when pancreatic β-cells fail to secrete sufficient amounts of insulin to meet the metabolic demand. In the natural history of type 2 diabetes, pancreatic β-cells initially compensate for insulin resistance by hypersecretion of insulin, but with time, progressive β-cell failure leads to insulindeficiency and hyperglycemia ensues. Progression in diabetes leads to the development of chronic complications such as blindness, kidney failure, neuropathy, cardiovascular diseases and amputations. Thus novel ways to prevent and treat type 2 diabetes are urgently needed.

Non-pharmacological approaches including diet modification, weight control, regular exercise and patient education are used as first-line therapy for the management of type 2 diabetes and remain important for optimization of metabolic control. When lifestyle modification fails to achieve or sustain adequate glycemic control, insulin or oral anti-diabetic agents are typically used to manage the disease. Treatment options with oral agents are quite diverse, including metformin (Glucophage®) (inhibition of hepatic glucose production), thiazolidinediones (TZDs) (insulin sensitizers), α-glucosidase inhibitors (inhibition of gut glucose absorption) and sulphonylureas (β-cell secretagogues). Several new drugs with glucose-lowering efficacy offering certain advantages have recently become available, such as injectable glucagon-like peptide-1 (GLP-1) receptor agonists and oral dipeptidyl peptidase-IV (DPP-IV) inhibitors. Both of them are promising not only to normalize fasting and postprandial glucose levels but also to improve β-cell functioning and mass by stimulating neogenesis. Indeed, β-cell plasticity enables these cells to adapt their number and volume (β-cell mass) and their function to the increased secretory demand linked to insulin resistance. The currently available classes of oral agents differ in mechanism and duration of action, the degree to which they lower blood glucose and their side-effect profile. Although in recent years the emphasis on initial therapy has been shifting from insulin secretagogues to insulin sensitizers, their mechanisms of action are still incompletely understood. Exciting recent developments have shown that AMP-activated protein kinase (AMPK), a phylogenetically conserved serine/threonine protein kinase, is one of the probable target of major antidiabetic drugs, metformin and TZDs, and of insulin sensitizing adipokines (e.g., adiponectin). Evidence accumulated over the past few years indicates that AMPK acts as an integrator of regulatory signals monitoring systemic and cellular energy status, thus providing powerful validation of the concept of targeting the AMPK pathway for the treatment of type 2 diabetes.

3 Managing type 2 diabetes by targeting AMPK pathway: an emerging concept

3.1 Lifestyle intervention strategies to prevent and control type 2 diabetes

During the past two decades, type 2 diabetes has reached pandemic proportions in association with rising levels of obesity and inactivity. Although physical activity is known to produce multiple health benefits, many people are considered to be relatively physically inactive. Current guidelines recommend practical, regular and moderate regimens of physical activity. The multiple metabolic adaptations that occur in response to physical activity can improve glycemic control for individuals with type 2 diabetes or delay the onset of the disease. This is supported by observational studies and clinical trials of diet, drugs or exercise, in persons at high risk for type 2 diabetes (3–5). The Diabetes Prevention Program (DPP) Research Group conducted a large, randomized clinical trial involving adults in the United States who were at high risk for the development this disease (5). Eligible participants were randomly assigned to one of three interventions: standard lifestyle recommendations plus metformin at a dose of 850 mg twice daily, standard lifestyle recommendations plus placebo twice daily, or an intensive

program of lifestyle modification (low-calorie, low-fat diet and physical activity of moderate intensity). The incidence of diabetes was reduced by 58% with the lifestyle intervention and by 31% with metformin, as compared with placebo after 3 years of follow-up. These effects were similar in men and women. In this study, the lifestyle intervention was particularly effective (and more than metformin treatment), with one case of diabetes prevented per seven persons treated for three years.

In addition to impacting development of type 2 diabetes, regular physical activity participation is associated with numerous health benefits for the individual with diabetes. Physical activity appears to have an independent and beneficial effect on insulin action, glycemic control and metabolic abnormalities associated this disease (6). Regular physical activity leads to a number of beneficial physiological changes that favourably affect muscle and liver insulin sensitivity, muscle glucose uptake and utilization and overall glycemic control (7). In addition, performed activity can improve lipid profile, decrease body weight and percentage of body fat, lower blood pressure, positively affect thromboembolic state and thus reduce overall cardiovascular disease risk (8,9). These beneficial effects are partly explained by AMPK activation during physical activity not only in skeletal muscle but also in liver and adipose tissue (10). Indeed, in type 2 diabetes patients, improvement in glycemic control during physical activity is linked to enhanced glucose transport in skeletal muscle and reduction in hepatic glucose production (11). Interestingly, these effects are insulin-independent. This probably explains that, despite whole-body insulin resistance, AMPK can be fully activated by physical training in type 2 diabetes patients as compared to healthy controls (12). In addition to this acute metabolic effect on glucose disposal, repeated physical activity improves insulin action in skeletal muscle from obese and insulin-resistant individuals (13). This improvement parallels an increase in the oxidative capacity of skeletal muscle by up-regulating lipid oxidation and the expression of proteins involved in mitochondrial biogenesis. Both of these mechanisms are linked to AMPK activation that seems to be a crucial key for metabolic adaptation to physical activity (see below). Use of AMPK agonists could be a new strategy to increased endurance without exercise in physically inactive type 2 diabetes patients.

3.2 Management of β-cell mass and function in type 2 diabetes

Clinical studies demonstrated that TZDs improve β-cell function for a long time both as monotherapy and in combination with metformin or sulfonylurea (14). They may also improve insulin processing, as demonstrated by a reduction in the proinsulin/total immunoreactive insulin ratio, (an indicator of β-cell dysfunction) whereas sulfonylureas did not (15). More importantly, TZDs can prevent or delay type 2 diabetes in patients with impaired fasting glucose. This has been clearly demonstrated in different clinical studies, such as TRIPOD (Troglitazone in Prevention of Diabetes) (16), PIPOD (Pioglitazone in Prevention of Diabetes) (17) in women with prior gestational diabetes mellitus, DPP (5) and DREAM (Diabetes Reduction Assessment with Ramipril and Rosiglitazone Medication) (18). In consequence, these clinical trials support the idea that early detection and proper preventive measures, including TZDs treatment, can prevent the progression of high-risk patients from developing DM2. Interestingly, metabolic and insulin-sensitizing effects of TZDs have been shown to be in part mediated through adiponectin-dependent activation of AMPK since activation of AMPK by rosiglitazone treatment is diminished in adiponectin KO mice (19). TZDs can markedly enhance the expression and secretion of adiponectin in humans and rodents *in vitro* and *in vivo*, through the activation of its promoter and also antagonize the suppressive effect of TNF-α on the production of adiponectin (20).

3.3 Management of cardiovascular diseases in type 2 diabetes

Cardiovascular disease (CVD) is the main cause of morbidity/mortality in diabetes. As suggested by the results of the clinical trial ACCORD (Action to Control Cardiovascular Risk

in Diabetes), there is no evidence that normoglycemia can, by itself, lower the CVD in type 2 diabetic patients (21). Thus, it is necessary to develop new strategies to reduce CVD in this population. Metformin has cardioprotective effects on lipids, thrombosis and blood flow. It was reported that metformin was associated with a reduction by 32% in any diabetes related endpoint and in myocardial infarction by 39% (22). The figures for macrovascular complications compare favourably for those described for other cardioprotective agents such as angiotensin converting enzyme (ACE) inhibitors and statins. The beneficial effect of metformin on CVD is independent of changes in insulin resistance or glucose control suggesting a direct effect on vessels. Studies *in vitro* or in type 2 diabetes rodents models clearly demonstrated that metformin normalizes endothelial function (23). Similarly, adiponectin has protective effect on endothelial functions and CVD and type 2 diabetes mellitus are associated with low plasma concentration of adiponectin (24). In isolated rat hearts, adiponectin protects from myocardial contractile dysfunction and limits infarct size in following ischaemia and reperfusion by a mechanism involving activation of AMPK and production of NO (25). In humans, clinical trials concerning use of recombinant adiponectin during myocardial infarction are not available. Nevertheless, the increase of blood adiponectin levels to normal range after a cardiovascular event could be a new therapeutic target. To this aim, the association of drugs classically used for the prevention of recurrence of CVD and some dietary modifications could be useful to restore adiponectin levels in the normal range. This has been described recently in a double-blind, placebo-controlled, parallel trial where 44 patients who survived myocardial infarction and received statin therapy for at least 6 months were randomised to receive either 3 × 85 mg/day of chokeberry flavonoid extract or placebo for a period of 6 weeks (26). A significant increase in adiponectin levels and also a reduction in inflammatory markers were found when flavanoid extract was used. These beneficial effects, regardless of statins, are potentially interesting for secondary prevention of ischaemic heart disease.

4 Structure and regulation of AMPK

AMP-activated protein kinase (AMPK) plays an important role in the regulation of cellular and whole-body energy homeostasis. AMPK has been described as a "metabolic master switch" that mediates the cellular adaptation to nutritional and environmental variations depleting intracellular ATP levels, including heat shock, hypoxia, starvation or prolonged exercise. Regardless, the result of AMPK activation is the inhibition of energy-consuming biosynthetic pathways (such as fatty acid synthesis in liver and adipocytes, cholesterol synthesis in liver, protein synthesis in liver and muscle and insulin secretion from β-cell) and the activation of ATP-producing catabolic pathways (such as fatty acid uptake and oxidation in multiple tissue, glycolysis in heart and mitochondrial biogenesis in muscle). AMPK can also modulate transcription of specific genes involved in energy metabolism, thereby exerting long-term metabolic control.

AMPK is composed of three different subunits α, β and γ. Homologues of these subunits have been identified in mammals, *Drosophila*, worm, yeast, plants and primitive protozoon, with a high degree of conservation, suggesting the important role of AMPK in regulation of metabolic homeostasis. In mammals, the heterotrimeric complexes combine catalytic α subunit (α1 or α2), with β (β1 or β2) and γ (γ1, γ2 or γ3) regulatory subunits encoded by separate genes yielding to 12 heterotrimeric combinations (Figure 1). In addition, the γ2 and γ3 genes also give rise to short and long splice variants adding to the diversity. Differences in level expression and tissue distribution of the three subunits types were also described. The catalytic α1 subunit is strongly expressed in the kidney, the lung and the adipose tissue, whereas catalytic α2 subunit is predominantly found in heart and skeletal muscles. The regulatory β1 subunit is preferentially expressed in the liver and β2 in the skeletal muscle. The regulatory γ1 and γ2 subunits have broad tissue distribution whereas γ3 seems highly specific to glycolytic skeletal muscle. Moreover, despite the possible multiple combinations of the different subunits, only three are

found in human skeletal muscle (α2β2γ1; α2β2γ3 and α1β2γ1) with different levels of expression and activation during exercise (27,28). The α subunit contains a serine/threonine protein kinase catalytic domain in the N-terminal part, typical of the protein kinase superfamily (29). The catalytic domain has a site of phosphorylation at threonine residue Thr172 within the activation loop (T-loop) which is the key site for AMPK activation by upstream kinases (30,31). In the extreme C-terminus part, a region of ~150 amino acid residues is required for association with β and γ subunits, whereas the central part seems to have an inhibitory function (32,33). Moreover, localization of α2-containing complexes both in the nucleus and the cytoplasm implies a direct control of genes expression *via* phosphorylation of transcription factors (34). The β subunit appears to stabilize the interaction between α and γ subunits through its binding domain in the C-terminal part (35,36). In addition, the presence of a glycogen-binding domain in the N-terminal part corroborates the fact that AMPK binds glycogen granules and regulates glycogen metabolism (37,38). The high variable γ subunits are characterized by the presence of four cystathionine-β synthase (CBS) domains organized in tandem pairs to generate two binding sites for AMP or ATP, bound in a mutually exclusive manner, called Bateman domains (39). Glycogen storage disorders and a related hereditary heart disease have been described consecutively to mutations in these domains for the γ3 and γ2 genes, respectively (40,41). Recently, in addition to the yeast homologue Snf1 (42) and mammalian glycogen-binding site of β1 subunit (43), crystal structure of a trimeric complex containing the C-terminal domains of α1 and β2 with full-length γ1 was resolved, in presence of AMP or Mg-ATP. The results confirm the binding of either AMP or ATP on two known sites of the γ subunit and reveal a third site with tightly bound AMP. Under physiological conditions, AMPK interacts predominantly with Mg-ATP to form inactive complexes which are more abundant than complexes with AMP. These studies also suggest that binding of phosphorylated α and/or β subunit(s) would be possible in the presence of AMP but not when ATP is bound to the γ subunit (44). In addition to the multiple combinations of the three different subunits in the heterotrimeric complex, as well as their tissue-specific expression, the various regulations (i.e. phosphorylation, binding of AMP/ATP and glycogen) and subcellular localization may constitute a very fine and specific regulation of the metabolic tracts by AMPK.

Regulation of AMPK activity involves both direct allosteric activation by AMP and reversible phosphorylation of AMPKα subunit on Thr172 by upstream kinases (Figure 2). The combination of the allosteric and phosphorylation effects causes >1000-fold increase in kinase activity (45) allowing to respond to small changes in cellular energy status in a highly sensitive manner. Under conditions of high cellular energy demands, intracellular ATP is reduced, AMP levels rise and the AMP/ATP ratio forms a very sensitive indicator of cellular energy status (46). Binding of AMP to the regulatory γ-subunit of AMPK promotes allosteric activation, phosphorylation of Thr172 by upstream kinases and inhibition of dephosphorylation of Thr172 by protein phosphatases. There are at least two protein kinases capable of phosphorylating Thr172 *in vivo*, LKB1 (47–49) and Ca2+/calmodulin-dependent kinase kinase (50–52), especially the β isoform (CaMKKβ). LKB1, originally identified as a tumour suppressor, exists as a complex with two accessory subunits, STRAD and MO25. The LKB1 complex is supposed to be constitutively active and to promote activation by the AMP-dependent pathway (49) but recent studies indicate that cytosolic localization and activity of LKB1 can be governed by LKB1 acetylation status in the liver (53). An alternate pathway for AMPK activation involves CaMKKβ which responds to changes in cytoplasmic Ca2+ levels, indicating that AMPK may be activated in the absence of increase levels of AMP (50–52). Phosphorylation and activation of AMPK can be reversed by protein phosphatases. Binding of AMP to AMPK induces a conformational change in the kinase domain that protects AMPK from dephosphorylation of Thr-172 (54), probably catalysed by a form of protein phosphatase-2C (55).

5 Mimicking the beneficial effects of physical exercise

Although appropriate diet and exercise regimes should therefore be the first choice of treatment and prevention of type 2 diabetes, there are patient groups for whom such regimes are not appropriate for other medical reasons, or where compliance is difficult because of social factors or poor motivation. In these cases, drugs acting on the signaling pathways that induce the favourable changes in whole body metabolism are attractive candidates for treatment and prevention. It is now well established that muscle contraction is a prototypical AMPK activator (56). It has been suggested that AMPK activation may recapitulate some of the exercise-induced adaptations and is likely to mediate beneficial effects of exercise on insulin sensitivity and glucose transport in skeletal muscle (57). Activation of AMPK with the pharmacological compound AICAR (5-Aminoimidazole-4-carboxamide-1-β-D- ribonucleoside, metabolized to ZMP which is an analog of AMP) increases running endurance in untrained mice suggesting that AMPK agonists are exercise mimetics (58). Furthermore, AICAR upregulates genes linked to oxidative metabolism, angiogenesis and glucose sparing (58). Thus, it is expected that part of the effect of physical activity in preventing the development of diseases related to a sedentary lifestyle is due to activation of AMPK. However, although the activation of AMPK in skeletal muscle can lead to the stimulation of glucose transport, contraction-stimulated glucose transport is normal or partly affected in transgenic mouse models overexpressing dominant negative form of AMPKα2, in skeletal muscle and in whole body AMPKα1 and AMPKα2 knock-out (KO) mice (59–62). These results suggest that muscle contraction leads to the activation of multiple redundant signaling pathways and that inhibition of only one is not sufficient to alter contraction-induced glucose transport. Conversely, activation of AMPK with agents that elicit or mimic the effects of contraction (changes in AMP:ATP ratio, intracellular Ca^{2-} levels and reactive oxygen species) is responsible for increase in muscle glucose transport. Studies with AMPK activators in animal models of type 2 diabetes have provided promising results. The first evidence came from *in vivo* treatment with the pharmacological compound AICAR of various animal models of insulin resistance, causing improvement in most, if not all, the metabolic disturbances of these animals (63–67). Interestingly, long-term AICAR administration prevents the development of hyperglycemia in Zucker diabetic fatty (ZDF) rats, improves peripheral insulin sensitivity in skeletal muscle and delays β-cell dysfunction associated with type 2 diabetes (66). In addition, ablation of AMPKα2 specifically in skeletal muscle, exacerbates the development of insulin resistance and glucose intolerance caused by high-fat feeding (68). Finally, it was reported that the insulin sensitizing effects of AICAR were diminished by inhibition of AMPK in C2C12 myotubes (69). Repetitive pharmacological activation of AMPK *in vivo* results in expression of specific muscle proteins mimicking some of the effects of exercise training such as increased glucose uptake and mitochondrial biogenesis (Figure 3). In rodent, AICAR or chronic intake of the creatine analogue β-guanidinopropionic acid (β-GPA) increases the expression of genes encoding glucose transporter GLUT4, hexokinase II and markers of mitochondrial biogenesis (70–73). A recent report reveals that peroxisome proliferator-activated receptor-γ coactivator-1 α (PGC-1α) expression is required for AMPK-dependent activation of gene expression, including PGC-1α itself, GLUT4 and mitochondrial genes (74). Interestingly, these gene expression effects are abolished in AMPKα2 KO mice and transgenic mice overexpressing a kinase-dead AMPKα2 mutant in skeletal muscle (mAMPK-KD mice) (75,76).

Recent studies support the critical role of AMPK in the metabolic adaptation of skeletal muscle to exercise training. Over-expression of an active AMPKγ1R70Q mutant in skeletal muscle significantly rises the relative proportion of type IIa/x fibers, the expression of PGC-1α and the activity of mitochondrial markers in sedentary transgenic animals compared to their sedentary controls, without any further increase with exercise training (77). Moreover, AMPKα2 KO mice have a disturbed muscle energy balance during exercise, as indicated by a reduced ATP content (76). Physical training also increases circulating adiponectin and mRNA

expression of its receptors in muscle, which may mediate the improvement of insulin resistance in response to exercise by activation of AMPK (78). Interestingly, metformin treatment of subjects with type 2 diabetes significantly increases AMPK activity in skeletal muscle and this stimulation is accompanied by enhanced peripheral glucose disposal (79,80). Metformin is also able to restore glucose uptake stimulation in insulin-resistant cardiomyocytes, suggesting that AMPK activation could be a potential therapeutic approach to treat insulin resistance in diabetic hearts (81). Finally, activation of AMPK in response to physical exercise is also observed in extra-muscular tissues such as liver and adipose tissue and might account for additional metabolic benefits (10).

6 Mimicking the beneficial effects of calorie/dietary restriction

Calorie intake is an important determinant of health. Excessive calorie intake and subsequent abdominal obesity increase the risk of developing chronic disease such as type 2 diabetes, cardiovascular complications and premature mortality. In overweight and obese humans, calorie restriction (CR) with adequate nutrition improves glucose tolerance and insulin action and reduces mortality for type 2 diabetes and cardiovascular diseases (82–85). Even if calorie restriction produces a metabolic profile desirable for treating type 2 diabetes, it is unlikely that such restriction will be widely adopted because of the difficulty in maintaining long-term low calorie intake in modern society. There is an increased interest in developing pharmacological agents acting as "calorie-restriction" mimetics. Such agents could provide the beneficial metabolic, hormonal and physiological effects of calorie restriction without altering dietary intake or experiencing any potential adverse consequences of excessive restriction. The plant-derived polyphenolic compounds, such as resveratrol (RSV) present in grapes, peanuts and several other plants, recently held great attention for their role in mimicking the effects of calorie restriction. This was evidenced by the findings that RSV can delay the aging process in lower eukaryotes (86). In rodents, RSV prevents the deleterious effects of excess calorie intake on insulin resistance and metabolic disorders (87–91). RSV acts as a potent activator of the NAD(+)-dependent deacetylases sirtuins including SIRT1, one of the seven mammalian sirtuin genes. SIRT1 has been suggested to prime the organism for metabolic adaptation to insulin resistance, increasing hepatic insulin sensitivity and decreasing whole-body energy requirements (91,92). It is also involved in insulin secretion (93,94) and lipid mobilization (95). Interestingly, the polyphenols RSV and epigallocatechin-3-gallate (EGCG) were recently identified as potent activators for AMPK *in vitro* and *in vivo* (88,96,97). Furthermore, it has been demonstrated that SIRT1 functions as an upstream regulator of the LKB1/AMPK signaling axis in response to RSV activation in hepatocytes (98). SIRT1 promotes LKB1-dependent AMPK stimulation through the direct deacetylation and activation of LKB1 (53). It should be noted that the regulation of LKB1/AMPK signaling by SIRT1 is probably tissue-specific as resveratrol-stimulated AMPK activation is independent of SIRT1 in neurons (99). This is consistent with *in vivo* data suggesting that resveratrol may act on additional sirtuins than SIRT1 or on different targets (92). Nevertheless, understanding the role of AMPK in the action of polyphenols will provide valuable information to aid decisions about whether these compounds might be used as additives in foods or beverages to promote health and attenuate, or delay, the onset of various diseases, including cardiovascular disease and diabetes.

7 Role of AMPK in the control of glucose homeostasis

Glucose homeostasis is maintained by a balance between glucose production and glucose uptake by peripheral tissues. Elevated hepatic glucose production (HGP) is a major cause of fasting hyperglycemia in diabetic subjects (2). The importance of AMPK in the control of glucose output by the liver is emphasized by findings showing that pharmacological activation of AMPK leads to inhibition of HGP *in vitro* and *in vivo*. It has been first shown that systemic infusion of AICAR in normal and insulin-resistant obese rats led to the inhibition of hepatic

glucose production (HGP) (63). Similarly, it was reported that AMPK activation by metformin in cultured rat hepatocytes mediates the inhibitory effects of the drug on hepatic glucose production (100). There is now good *in vivo* evidence from studies of mice deficient in the upstream kinase LKB1 in the liver that the blood-lowering effect of metformin is mediated by activation of the LKB1/AMPK axis (101). It has been also reported that short-term hepatic expression of a constitutively active form of the α2 catalytic subunit (AMPKα2-CA) led to mild hypoglycemia in normal mice (102,103) and abolished hyperglycemia in diabetic *ob/ob* and STZ-induced diabetic mice (102). This hypoglycemic effect of AMPK activation is consistent with the abolition of HGP, as suggested by the down-regulation of gluconeogenic gene expression (e.g., phosphoenolpyruvate carboxykinase [PEPCK] and glucose-6-phosphatase [G6Pase]) and inhibition of glucose production in hepatocytes expressing AMPK-CA or treated with AICAR (102–104). Inhibition of gluconeogenesis by AMPK is achieved at least to a large extent *via* the regulation of a transcriptional coactivator, transducer of regulated CREB activity 2 (TORC2) (105). TORC2 mediates CREB-dependent transcription of PGC1α and its subsequent gluconeogenic targets PEPCK and G6Pase. TORC2 is regulated by multiple signaling pathways in response to changes in glucagon and insulin levels or intracellular energy status. AMPK activation causes TORC2 phosphorylation and sequesters the coactivator in the cytoplasm, thus blunting the expression of the gluconeogenic program (Figure 4). A physiological link has been established between the potent effects of various circulating adipocyte-derived hormones circulating levels and hepatic AMPK activity in the maintenance of blood glucose levels. Low blood glucose levels and reduced HGP in mice lacking resistin are likely related, at least in part, to activation of AMPK and decreased expression of gluconeogenic enzymes in the liver (106). Administration of adiponectin is known to reduce both blood glucose levels and expression of gluconeogenic genes. It has been shown that these effects required AMPK activation at least in the liver (107). According to this result, adiponectin failed to regulate HGP in liver-specific AMPKα2 KO mice (108).

Skeletal muscle is the main site for glucose disposal in the body. Insulin increases glucose uptake in the muscle by stimulating the translocation of glucose transporter GLUT4 from intracellular vesicles to the cell surface. (Figure 3). It has been shown that muscular AMPK activation stimulates muscle glucose uptake either by exercise or by AICAR and this occurs through a distinct mechanism than the insulin-signaling pathway. AICAR can effectively increase glucose transport and GLUT4 translocation in skeletal muscle, not only in lean subjects but also in type 2 diabetes subjects (109). An AMPK-dependant increase in glucose transport is therefore observed in insulin-resistant skeletal muscle, both in rodents and humans, providing evidence that the AMPK pathway can be activated in this case (46). Stimulation of AMPK in the muscle could be an efficient method to increase glucose uptake in an insulin-independent manner, thus bypassing defective insulin signaling, such as one observed in type 2 diabetes patients. Although the upstream stimuli that activate AMPK are relatively well known (46), the signaling mechanisms downstream of AMPK which regulate muscle glucose transport are not so well understood. It has been recently discovered that the Akt substrate of 160kDa (AS160/TBC1D4), downstream target of Akt, played a major role in regulating insulin-stimulated glucose uptake. Another possible downstream effector of AMPK in the regulation of muscle glucose transport is an AS160/TBC1D4 homolog, TBC1D1 (110). AS160/TBC1D4 is a Rab-GTPase activating protein that regulates the translocation of GLUT4 from intracellular vesicles to the plasma membrane by maintaining small G-proteins, known as Rab, in a GDP-bound state. The phosphorylation of AS160 at specific sites is thought to disrupt GTPase activity and allow the release of AS160 inhibition on GLUT4 vesicle intracellular localization, thus initializing the vesicle translocation (110). TBC1D1 is also a Rab-GTPase activating protein and its phosphorylation is enhanced in mouse skeletal muscle myotubes following treatment with AICAR (111). In a similar way, exercise and AICAR-induced AMPK activation caused AS160/TBC1D4 phosphorylation in skeletal muscle like insulin (28). Furthermore, AS160 phosphorylation as well as glucose uptake is increased in

skeletal muscle following a resistance exercise in humans. A positive correlation between increased AS160/TBC1D4 phosphorylation and muscle glucose uptake during post-exercise recovery has been observed, as well as increased muscle AMPKα2 activity (112). Taken together, these results show that AMPK-induced muscle glucose uptake stimulation is mediated by AS160/TBC1D4. This represents a point of convergence connecting insulin, contraction and AMPK-stimulated glucose transport. It has been observed that exercise-induced AMPK activation in muscle is diminished in both obese non-diabetic and obese type 2 diabetes subjects, but maintained in lean type 2 diabetes patients (12,113). This suggests that dysregulation of the AMPK pathway may be more associated with obesity rather than with type 2 diabetes *per se*. This reflects impaired adaptability to utilize lipid and carbohydrate fuels and to transition between them, also referred as metabolic inflexibility (114), observed in obese and insulin resistant people. It is therefore likely that obese patients may require a more intense exercise protocol to achieve the same benefits than in lean individuals (113). Consistently, in obese type 2 diabetes subjects, AS160 phosphorylation is blunted in skeletal muscle following moderate intensity exercise (113).

Several cytokines have been shown to stimulate glucose transport in muscle in an AMPK-dependent manner. Leptin is an adipokine recently shown to improve insulin action in muscle of patients with lipodystrophy, although the role of AMPK activation as mediator is not studied (115). Leptin is also known to stimulate glucose uptake in peripheral tissue (116,117). It has been shown that leptin selectively stimulated AMPKα2 phosphorylation and activation in skeletal muscle, further confirming the role of AMPK in leptin-mediated stimulation of glucose transport (118). Adiponectin also increases glucose transport in both lean and obese skeletal muscle, although the effect is less significant in the latter (119). This suggests a possible development of adiponectin resistance related to metabolic inflexibility observed in skeletal muscle from obese individuals. Interleukin-6 (IL-6) is a proinflammatory cytokine that activates AMPK and is thought to modify insulin sensitivity. Recently, it has also been recognized as a "myokine", due to its release from the skeletal muscle during prolonged exercise (120). Muscle strips obtained from healthy young men treated with IL-6 show an increased glucose uptake concomitant with AMPK phosphorylation (121). It has been shown that IL-6-stimulated glucose transport is mediated *via* the LKB1/AMPK/AS160 pathway. However, the same study also shows that IL-6 has a dual effect: short term IL-6 treatment is additive to insulin on activating glucose transport and AS160 phosphorylation, resulting in an improved glucose tolerance and insulin sensitivity in mice, whereas chronic exposure produces insulin resistance both *in vitro* and *in vivo* (122). It has been shown that AMPK participates in the regulation of IL-6 release from oxidative muscle. It has been also suggested that AICAR, in addition to activating AMPK, suppresses IL-6 release by an AMPK independent mechanism (123). Collectively these results demonstrate that AMPK plays a major role in glucose homeostasis by modulating glucose transport in skeletal muscle. Thus, AMPK could have a significant role in the modification of glucose muscle metabolism, thus establishing the AMPK pathway as an attractive target for the treatment of type 2 diabetes.

8 Role of AMPK in the control of lipid metabolism

Both insulin resistance and type 2 diabetes are characterized by dyslipidemia, which is an important and common risk factor for cardiovascular disease. Diabetic dyslipidemia is a cluster of potentially atherogenic lipid and lipoprotein abnormalities that are metabolically interrelated. AMPK coordinates the changes in the hepatic lipid metabolism and, so, regulates the partitioning of fatty acids between oxidative and biosynthetic pathways. Thus, once activated, AMPK phosphorylates and inactivates a number of metabolic enzymes involved in ATP-consuming cellular events, such as 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) and acetyl-CoA carboxylase (ACC), key enzymes in hepatic cholesterol and fatty acid synthesis (Figure 4). In addition, AMPK suppresses expression of

lipogenesis-associated genes such as fatty acid synthase, pyruvate kinase and ACC (102, 124–127). ACC is an important rate-controlling enzyme for the synthesis of malonyl-CoA, which is both a critical precursor for the biosynthesis of fatty acids and a potent inhibitor of mitochondrial fatty acid oxidation. Inhibition of ACC by AMPK leads to a drop in malonyl-CoA content and a subsequent decrease in fatty acid synthesis and increase in fatty acid oxidation, thus reducing excessive storage of triglycerides. Malonyl CoA decarboxylase (MCD), an enzyme involved in the turnover of malonyl-CoA, has been shown to be activated by AMPK in response to energy depletion, resulting in reduced malonyl CoA levels and increased fatty acid oxidation (128). Consistently, overexpression of AMPKα2-CA in the liver, treatment with AICAR or metformin in lean and obese rodents increase plasma β-hydroxybutyrate levels, suggesting elevated hepatic lipid oxidation, concomitantly with a decrease in plasma triglyceride levels (63,100,102). Conversely, liver-specific AMPKα2 deletion leads to increased plasma triglyceride levels and enhances hepatic lipogenesis (108). These data emphasizes the critical role for AMPK in the control of hepatic lipid deposition *via* decreased lipogenesis and increased lipid oxidation, thus improving lipid profile in type 2 diabetes. In addition, AMPK also emerged as a key player in the regulation of fatty acid oxidation in skeletal muscle (Figure 3). In rodent skeletal muscle, stimulation of AMPK by AICAR increased the oxidation of palmitate (129,130). Leptin, an adipocyte-secreted hormone that plays a pivotal role in regulation of energy expenditure, was found to increase fatty acid oxidation in skeletal muscle by activating AMPK (118). Leptin activates AMPK by a dual effect, i.e. early and transient activation of AMPK by leptin directly at the level of muscle and a more sustained activation mediated through the hypothalamic-sympathetic nervous system axis and α–adrenergic receptors in muscle (118). Mice harboring a gain-of-function of the γ3 subunit (R225Q) in skeletal muscle demonstrate lower intramuscular TG content, increased lipid oxidation and protection against diet-induced insulin resistance (131). As in the liver, AMPK enhances fatty acid oxidation by inactivating ACC, thereby reducing the synthesis of malonyl CoA. However, recent studies using mAMPK-KD mice indicate that AMPK-independent pathways can regulate skeletal muscle fatty acid oxidation (132). Furthermore, incubation of isolated rat muscle with both AICAR and electrical stimulation result in higher fatty acid oxidation than for each condition alone (133). In human skeletal muscle, the connection between changes in AMPK activation and fatty acid oxidation during exercise is not clear (134,135).

9 Management of fatty liver disease by AMPK activation

One of the critical complications of type 2 diabetes is nonalcoholic fatty liver disease (NAFDL), a disorder of triacylglycerol accumulation in the liver that has potential to develop into end stage liver failure. It has been proposed that steatosis primed the liver to progress to more severe liver pathologies when individuals were exposed to subsequent metabolic and/or environmental stresses. Insulin resistance is a major feature of NAFDL and studies in humans and various animal models suggest that efforts to enhance insulin sensitivity might improve fatty liver disease. The efficacy of insulin-sensitizer metformin as a treatment for this disease is confirmed in obese ob/ob mice, which develop hyperinsulinemia, insulin resistance and fatty livers (136). Similarly, adiponectin treatment restores insulin sensitivity and decreases hepatic steatosis by lowering the triglyceride content in the liver of obese mice (137). Metabolic improvement of adiponectin is linked to an activation of AMPK in the liver that decreases fatty acid biosynthesis and increases mitochondrial fatty acids oxidation (138). This is confirmed by a decrease in liver triglycerides content in lean and obese rodents during AICAR infusion (63) and treatment with small-molecule AMPK activators (139). Increased intracellular fat content in liver associated with insulin resistance leads to the hypothesis that a mitochondrial dysfunction in substrate oxidation is a primary defect in insulin resistant. It was recently demonstrated that activation of AMPK by RSV protected against lipid accumulation in the liver of diabetic mice (88), in association with increased mitochondrial number (87) and SIRT1-

dependent deacetylation of peroxisome proliferator-activated receptor coactivator (PGC)-1α, a master regulator of mitochondrial biogenesis (87,140). Hepatocytes deleted for both AMPK catalytic subunits have reduced mitochondrial biogenesis as suggested by decreased transcript and protein expression of key mitochondrial constituents such as PGC-1α, cytochrome c oxidase I (COX I), COX IV and cytochrome c genes (141). These results emphasize the importance of AMPK in the regulation of cellular energy homeostasis through the control of adaptive mitochondrial function. However, the role of the AMPK system in the treatment of fatty liver diseases remains to be clearly established in humans. Its importance is strongly indicated by recent studies with AICAR infusion in type 2 diabetic patients. This study reported that AICAR infusion resulted in significant decline in circulating plasma non-esterified fatty acids (NEFA) levels, suggesting stimulation of hepatic fatty acid oxidation and/or reduction in whole body lipolytic rate (142).

10 Role of AMPK in the regulation of β-cell function

Insulin resistance and insulin secretion defects are major risk factors for type 2 diabetes (143). The pathogenesis of type 2 diabetes is associated with different degrees of β-cell failure relative to varying degrees of insulin resistance. A progressive decrease of β-cell function leads to glucose intolerance, which is followed by type 2 diabetes that inexorably aggravates with time (144). According to the glucolipotoxicity hypothesis (145), chronic high glucose dramatically influences β-cell metabolism and results in an increase of cytosolic fatty acyl-CoA partitioning toward potentially toxic cellular products (e.g., diacylglycerol, ceramide and lipid peroxides). This leads to impaired insulin secretory response to glucose and ultimately in apoptosis. Evidence from recent literature clearly demonstrated that changes in AMPK signaling are important in the pathogenesis of both β-cell glucolipotoxicity and type 2 diabetes. Metformin, TZDs and AICAR treatments favor fatty acid β-oxidation and prevent glucolipotoxicity-induced insulin secretory dysfunction in β-cells (146–148). These results have been confirmed by using adenovirus-mediated over-expression of AMPKα1-CA in β-cell (149). A recent study reported that AICAR dose-dependently improves β-cell function without changing intracellular TG levels and may act through reducing apoptosis induced by prolonged hyperglycemia. However, the role of AMPK in the control of β-cell death remains controversial (150–155).

In β-cells, AMPK activity is rapidly decreased by elevations in glucose concentration over the physiological range indicating that AMPK could play a role in insulin release acting as a fuel sensor (156–158). Activation of AMPK by AICAR, metformin, TZDs and berberine or by overexpression of AMPKα1-CA markedly reduces glucose-stimulated insulin secretion in β-cell lines and in rodent and human islets (149,156,159,160). Conversely, overexpression of a dominant-negative form of AMPK stimulates insulin release at low glucose concentrations (157,161). These data suggest that activation of AMPK inhibits insulin release to maintain glucose homeostasis. Thus, inhibition rather than activation of AMPK would be desirable for the treatment of type 2 diabetes in order to reverse the decline in glucose-induced insulin secretion. Surprinsingly, it has been reported that agents that suppress insulin secretion, such as diazoxide, improve glucose tolerance and β-cell function (162). This effect is thought to be mediated *via* hyperpolarization of β-cells, thereby providing β-cell rest by reducing insulin release and could protect against the negative effects of overstimulation. One important point to take into account in this context is the role of pancreatic AMPK during the adaptation of insulin secretion in front of various degrees of insulin resistance during the onset of type 2 diabetes. First, AMPK-mediated suppression of insulin release could directly counteracts glucolipotoxicity, which may prevent functional exhaustion of β-cells in prediabetic states. A decrease in β-cell mass is likely to play a role in the pathogenesis of human type 2 diabetes (163) as it does in rodent models of the disease (164). Second, inhibition of insulin release would reduce the pathological basal hyperinsulinemia and markedly increase insulin sensitivity

and hence improve β-cell function and mass. Consistently, systemic AICAR infusion in prediabetic Zucker fatty rats prevents the development of hyperglycemia and preserved β-cell mass (66).

11 Management of cardiovascular diseases by AMPK activation

Type 2 diabetes is associated with an increased risk of cardiovascular disease and coronary heart disease mortality. The high energy demands of the heart are primarily met by the metabolism of fatty acids and glucose, both processes being regulated by AMPK. Indeed, AMPK stimulates glycolysis and sustains energy supply during ischemic stress. Promotion of glucose oxidation or inhibition of fatty acid oxidation in ischemic/reperfused hearts could be a promising novel therapeutic approach to myocardial ischemic conditions. Such a mechanism has been demonstrated during the phenomenon called ischemic preconditioning. Brief episodes of myocardial ischemia render the heart more resistant to subsequent ischemic episodes (165). Ischemic preconditioning is known to induce endogenous protective mechanisms in the heart. It activates AMPK in a PKC-dependent manner and promotes glucose utilization in myocardial cells, supporting resistance toward ischemic consequences (166). Thus, AMPK activators could be of particular interest for the management of myocardial ischemia. Attractively, it has been reported that adiponectin protects the heart from ischemia by activating AMPK and increasing the energy supply to heart cells (167). In addition, it has also been reported that adiponectin attenuates cardiac hypertrophy through activation of AMPK signaling pathway (168,169).

The presence of endothelial cell dysfunction in patients with type 2 diabetes, as manifested by impaired vascular relaxation or increase in circulating vascular cell adhesion molecules is thought to be one component of the inflammatory process initiating atherogenesis. In this respect, metformin has been proposed to improve endothelium function in diabetes by favoring phosphorylation of endothelial NO synthase (eNOS) by AMPK activation (23). Metformin was also shown to relax endothelium-denuded rat aortic rings pre-contracted with phenylephrine, showing that AMPK can induce vasorelaxation in an endothelium- and NOS-independent manner (170). Thus, vascular AMPK could be involved in the metabolic regulation of vascular tone. AMPK activation in response to hypoxia or metabolic challenge can induce vasorelaxation of big vessels (171,172), thereby favoring blood flow. Interestingly, AMPK-dependent adiponectin vascular effects have been demonstrated for angiogenic repair in an ischemic hind limbs model (173).

12 Conclusion and medical perspectives

Because of its global favourable effects on energy metabolism pathways, it is tempting to consider AMPK as a potential therapeutic target in the prevention and the treatment of type 2 diabetes and insulin resistance. A large number of AMPK activators have been employed with promising results in diabetic animal models and these encouraging results have provided the rationale for the development of new pharmacological (e.g., A-769662) but also nutritional (e.g., polyphenols) AMPK activators. AMPK activation in the liver and skeletal muscle entails metabolic changes that are beneficial for the diabetic patients with inhibition of HGP and stimulation of glucose uptake in skeletal muscle which help to maintain glycemia. However, some of the effects of AMPK activation in other organs or tissues should be carefully evaluated. The widespread cellular functions of AMPK make its selective targeting in therapeutics a difficult one, with simultaneous advantageous and deleterious consequences being possible. Thus, care should be taken that other metabolic effects due to AMPK activation could be detrimental for diabetic patients. One of the major caveats in the use of AMPK activators is their possible role in the regulation of food intake. Stimulation of AMPK expressed in specific nuclei of the hypothalamus has been shown to increase food intake (174). Thus, the ideal

AMPK activators will be administrated by the oral route, activate AMPK at low concentration and be effective in specific target organs, such as the liver and skeletal muscle but not the hypothalamus, and will also have minimal off-targets. An emerging concept is the use of tissue-specific pharmacological activation of AMPK that could be achieved through isoform-specific activation of AMPK. Interestingly, the small AMPK activator A-769662 is mainly targeted to the liver (175) and treatment of animal models with this compound recapitulates many of the effects expected for a specific hepatic AMPK activation (102). Similarly, it appears that the major effect of intravenous AICAR infusion in type 2 diabetic patients is restricted to the liver with inhibition of hepatic glucose output and decreased blood glucose levels (142). Curiously, AMPK activation is not evident in muscle biopsies from healthy volunteers (176) or diabetic patients (142) in response to AICAR infusion. These human data raise the question about the effective dose in producing a detectable AMPK activation in skeletal muscle and the benefit in term of glucoregulation in diabetic patients.

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Abbreviations

ACC
: acetyl CoA carboxylase

AICAR
: 5-aminoimidazole-4-carboxamide riboside

AMPK
: AMP-activated protein kinase

HGP
: hepatic glucose production

HMG-CoA
: hydroxy-3-methylglutaryl-CoA

KO
: knockout

PGC-1α
: peroxisome proliferator-activated receptor-γ coactivator-1α

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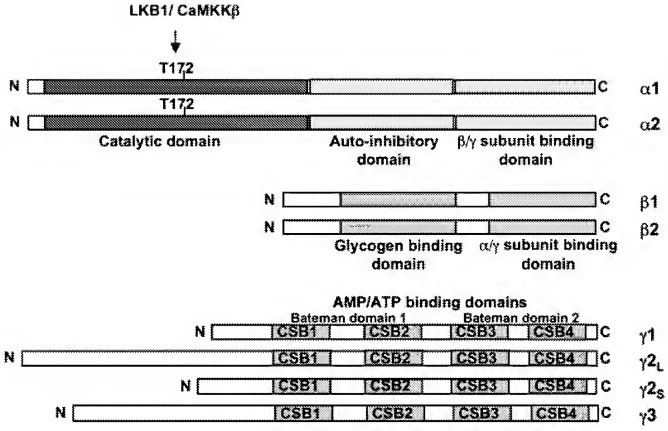

Figure 1. Structure of AMPK

The mammalian AMPKα (α1 and α2), AMPKβ (β1 and β2) and AMPKγ (γ1, γ2 short form, γ2 long form and γ3) subunits are shown. The α-subunits contains the Thr172 residue that must be phosphorylated (P) by upstram kinases for activity and an autoinhibitory sequence domain that inhibits the activity of the kinase domain. The C-terminal domain is required for binding the β- and γ-subunits. The β-subunits contains central glycogen-binding domains and C-terminal domain that is required for binding the α- and γ subunits. The three γ-subunit isoforms have variable N-terminal domains and four conserved cystathionine beta-synthase motifs (CBS1–4). The CBS motifs act in pairs to form two Bateman domains that bind AMP or ATP.

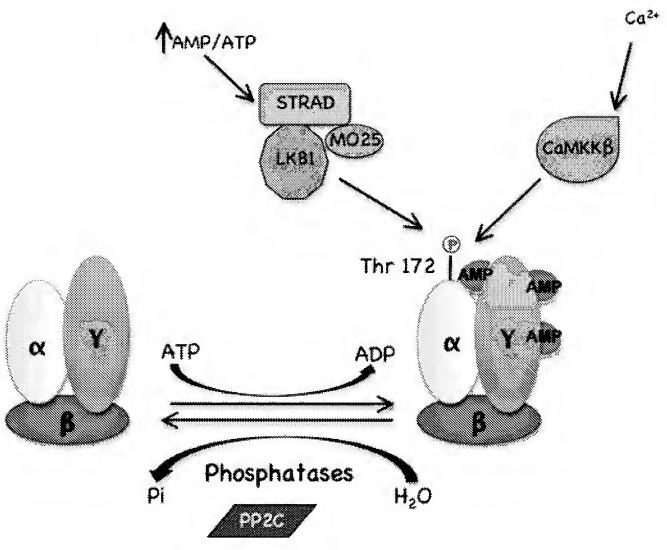

Figure 2. Regulation of AMPK activation

AMPK is activated by phosphorylation of Thr 172 catalysed by LKB1:STRAD:MO25 complex in response to increase in the AMP/ATP ratio and by CaMKKβ in response to elevated Ca^{2+} levels. Thr172 is dephosphorylated by PP2C protein phosphatase switching active AMPK to the inactive form.

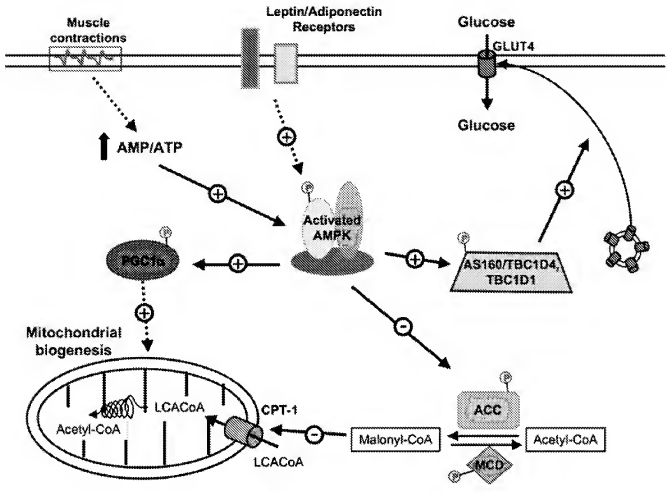

Figure 3. AMPK and the regulation of hepatic metabolism

Activation of AMPK leads to the inhibition of cholesterol synthesis by the phopshorylation of HMG-CoA reductase. By inhibiting ACC and activating MCD, AMPK increases fatty acid oxidation via the regulation of malonyl CoA levels, which is both a critical precursor for biosynthesis of fatty acids and a potent inhibitor of CPT-1, the shuttle that controls the transfer of LCACoA into the mitochondria. AMPK inhibits hepatic glucose production via the phosphorylation of TORC2 and inhibition gene expression for key gluconeogenic enzymes, G6Pase and PEPCK, and for the transcriptional co-activator PGC-1α. ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CPT1-α, carnitine palmitoyl transferase-1; G6Pase, glucose-6-phosphatase; LCACoA, Long Chain acyl CoAs; MCD, malonyl-CoA decarboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PGC1α, PPARγ co-activator 1α; TORC2, transducer of regulated CREB activity 2.

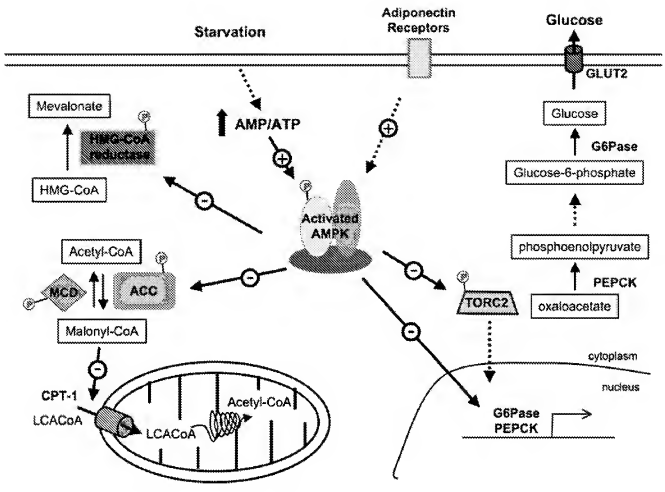

Figure 4. AMPK and the regulation of skeletal muscle metabolism

Proposed model for the role of AMPK in the regulation of lipid and glucose metabolism in skeletal muscle. AMPK activity may be increased by an altered energy nucleotide or by hormonal action. This activation of AMPK may result in an increase in glucose transport as well as an increase in fatty acid oxidation. ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; AS160, Akt substrate of 160kDa; CPT1-α, carnitine palmitoyl transferase-1; Glut4, glucose tranporter 4; MCD, malonyl-CoA decarboxylase; PGC1α, PPARγ co-activator 1α; LCACoA, Long Chain acyl CoAs.

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Cell Press Links

Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome.

Cool B, Zinker B, Chiou W, Kifle L, Cao N, Perham M, Dickinson R, Adler A, Gagne G, Iyengar R, Zhao G, Marsh K, Kym P, Jung P, Camp HS, Frevert E.

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AMP-activated protein kinase (AMPK) is a key sensor and regulator of intracellular and whole-body energy metabolism. We have identified a thienopyridone family of AMPK activators. A-769662 directly stimulated partially purified rat liver AMPK (EC50 = 0.8 microM) and inhibited fatty acid synthesis in primary rat hepatocytes (IC50 = 3.2 microM). Short-term treatment of normal Sprague Dawley rats with A-769662 decreased liver malonyl CoA levels and the respiratory exchange ratio, VCO2/VO2, indicating an increased rate of whole-body fatty acid oxidation. Treatment of ob/ob mice with 30 mg/kg b.i.d. A-769662 decreased hepatic expression of PEPCK, G6Pase, and FAS, lowered plasma glucose by 40%, reduced body weight gain and significantly decreased both plasma and liver triglyceride levels. These results demonstrate that small molecule-mediated activation of AMPK in vivo is feasible and represents a promising approach for the treatment of type 2 diabetes and the metabolic syndrome.

PMID: 16753576 [PubMed - indexed for MEDLINE]

Targeting the AMPK pathway for the treatment of Type 2 diabetes

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ELSEVIER Links FULL-TEXT ARTICLE

Diphenylene iodonium stimulates glucose uptake in skeletal muscle cells through mitochondrial complex I inhibition and activation of AMP-activated protein kinase.

Hutchinson DS, Csikasz RI, Yamamoto DL, Shabalina IG, Wikström P, Wilcke M, Bengtsson T.

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NADPH oxidase inhibitors such as diphenylene iodonium (DPI) and apocynin lower whole body and blood glucose levels and improve diabetes when administered to rodents. Skeletal muscle has an important role in managing glucose homeostasis and we have used L6 cells, C(2)C(12) cells and primary muscle cells as model systems to investigate whether these drugs regulate glucose uptake in skeletal muscle cells. The data presented in this study show that apocynin does not affect glucose uptake in skeletal muscle cells in culture. Tat gp91ds, a chimeric peptide that inhibits NADPH oxidase activity, also failed to affect glucose uptake and we found no significant evidence of NADPH oxidase (subunits tested were Nox4, p22phox, gp91phox and p47phox mRNA) in skeletal muscle cells in culture. However, DPI increases basal and insulin-stimulated glucose uptake in L6 cells, C(2)C(12) cells and primary muscle cells. Detailed studies on L6 cells demonstrate that the increase of glucose uptake is via a mechanism independent of phosphoinositide-3 kinase (PI3K)/Akt but dependent on AMP-activated protein kinase (AMPK). We postulate that DPI through inhibition of mitochondrial complex 1 and decreases in oxygen consumption, leading to decreases of ATP and activation of AMPK, stimulates glucose uptake in skeletal muscle cells.

PMID: 17391917 [PubMed - indexed for MEDLINE]

Interleukin-6 Regulation of AMP-Activated Protein Kinase

Potential Role in the Systemic Response to Exercise and Prevention of the Metabolic Syndrome

Neil B. Ruderman,[1] Charlotte Keller,[2] Ann-Marie Richard,[3] Asish K. Saha,[1] Zhijun Luo,[1] Xiaoqin Xiang,[1] Mercedes Giralt,[4] Vladimir B. Ritov,[5] Elizabeth V. Menshikova,[5] David E. Kelley,[5] Juan Hidalgo,[4] Bente K. Pedersen,[2] and Meghan Kelly[1]

Interleukin (IL)-6 is a pleiotropic hormone that has both proinflammatory and anti-inflammatory actions. AMP-activated protein kinase (AMPK) is a fuel-sensing enzyme that among its other actions responds to decreases in cellular energy state by enhancing processes that generate ATP and inhibiting others that consume ATP but are not acutely necessary for survival. IL-6 is synthesized and released from skeletal muscle in large amounts during exercise, and in rodents, the resultant increase in its concentration correlates temporally with increases in AMPK activity in multiple tissues. That IL-6 may be responsible in great measure for these increases in AMPK is suggested by the fact it increases AMPK activity both in muscle and adipose tissue in vivo and in incubated muscles and cultured adipocytes. In addition, we have found that AMPK activity is diminished in muscle and adipose tissue of 3-month-old IL-6 knockout (KO) mice at rest and that the absolute increases in AMPK activity in these tissues caused by exercise is diminished compared with control mice. Except for an impaired ability to exercise and to oxidize fatty acids, the IL-6 KO mouse appears normal at 3 months of age. On the other hand, by age 9 months, it manifests many of the abnormalities of the metabolic syndrome including obesity, dyslipidemia, and impaired glucose tolerance. This, plus the association of decreased AMPK activity with similar abnormalities in a number of other rodents, suggests that a decrease in AMPK activity may be a causal factor. Whether increases in IL-6, by virtue of their effects on AMPK, contribute to the reported ability of exercise to diminish the prevalence of type 2 diabetes, coronary heart disease, and other disorders associated with the metabolic syndrome remains to be determined. *Diabetes* 55 (Suppl. 2):S48–S54, 2006

Interleukin (IL)-6 is "a pleiotropic cytokine with a wide range of biological activities including immune regulation, hematopoiesis, inflammation, and oncogenesis" (1). It was initially viewed as a proinflammatory cytokine and because of its elevated plasma concentration in people with obesity and type 2 diabetes, it is generally thought to contribute to the low-grade inflammation present in these disorders. However, IL-6 may also protect against inflammation (2–7). AMP-activated protein kinase (AMPK) was first identified as a fuel-sensing enzyme, whose principal task is to assist cells in restoring cellular ATP when they are energy depleted as a result of glucose or O_2 deprivation or other stresses (8). However, like IL-6, its actions now appear to be somewhat more complex. In this article, we will review an increasing body of evidence that suggests a link between these molecules. In particular, we will address the notion that under certain circumstances, AMPK is activated by IL-6 and may mediate or modify some of its biological actions. We will also review the evidence that decreased AMPK activity could contribute to the development of obesity, dyslipidemia, and glucose intolerance (i.e., a metabolic-like syndrome) in IL-6 knockout mice.

AMPK

AMPK is a heterotrimer composed of a catalytic α-subunit and regulatory β- and γ-subunits. In humans, each subunit is encoded by either two or three distinct genes (α1, α2, β1, β2, γ1, γ2, γ3), so that there are 12 possible α, β, and γ combinations. All three subunits are necessary for full activity (9–11). AMPK is activated by decreases in the energy state of a cell as reflected by an increase in the AMP-to-ATP ratio. According to the most widely held view, increased binding of AMP to specific domains on the γ-subunit produces a conformational change that allows upstream kinases (AMPK kinases) to phosphorylate the threonine-172 residue of the α-subunit and activate the enzyme (10,11). Two AMPK kinases have recently been identified: LKB1, a tumor suppressor that is deficient in patients with the Peutz-Jegher syndrome (12,13), and a Ca^{2+}-dependent calmodulin-dependent protein kinase ki-

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ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CPT-1, carnitine palmitoyltransferase 1; FA-CoA, fatty acyl-CoA; IL, interleukin; MCD, malonyl-CoA decarboxylase.

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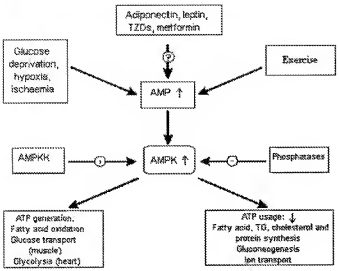

FIG. 1. AMPK activation and its effects on cellular energy state. AMPK activation concurrently leads to an increase in cellular processes that generate ATP and a decrease in processes that use ATP, but are not immediately necessary for cell survival. AMPK activation has been attributed to ATP depletion that leads to changes in the AMP-to-ATP ratio. Recent studies suggest that AMP binds to the γ-subunit of AMPK and produces a conformational change in the enzyme that makes it more susceptible to phosphorylation by an AMPK kinase (see text for details). Whether leptin and adiponectin, and pharmacological agents such as the thiazolidinediones, activate AMPK by altering the energy state of a cell, as does exercise (in skeletal muscle), is uncertain. The two AMPK kinases that have been identified to date are the tumor suppressor LKB1 and a calcium-dependent enzyme CAMKKβ. The precise role of changes in the activity of these enzymes and the role of phosphatases in this scheme are unknown. AMPKK, AMPK kinase; TZDs, thiazolidinediones. Adapted from Ruderman and Prentki (17).

nase, CAMKK (14–16). Numerous stimuli have been shown to activate AMPK (Fig. 1), including exercise, glucose deprivation, and hypoxia; various hormones including leptin, adiponectin, and catecholamines; and drugs such as metformin and thiazolidinediones (11,17). Likewise, a lack of leptin or its receptor, a surfeit of glucose, and the hormone ghrelin decrease AMPK activity in peripheral tissues (11). In many instances, it is unclear whether these hormones and drugs act by altering the energy state of the cell, by altering the functional activity of one of the AMPK kinases, or by an as yet undescribed mechanism.

Once it is activated, AMPK helps to restore the cell's energy state by acutely and chronically enhancing processes that generate ATP, such as fatty acid oxidation, and inhibiting others that consume ATP, but are not acutely necessary for survival (Fig. 1). The latter include protein, fatty acid, and triglyceride synthesis (9,10,17).

To illustrate how AMPK functions, a schema depicting some of its many effects on cellular fatty acid partitioning and metabolism is depicted in Fig. 2. AMPK increases fatty acid oxidation by diminishing the concentration of malonyl CoA, an allosteric inhibitor of carnitine palmitoyltransferase 1 (CPT-1), the enzyme that governs the transfer of long-chain fatty acyl-CoA (FA-CoA) from the cytosol into mitochondria. It accomplishes this acutely (minutes) by phosphorylating and inhibiting acetyl-CoA carboxylase (ACC), the rate-limiting enzyme for malonyl-CoA synthesis, and phosphorylating (most likely) and activating malonyl-CoA decarboxylase (MCD), which catalyzes malonyl-CoA degradation (18). In addition, AMPK subacutely (hours) represses the expression of the transcriptional

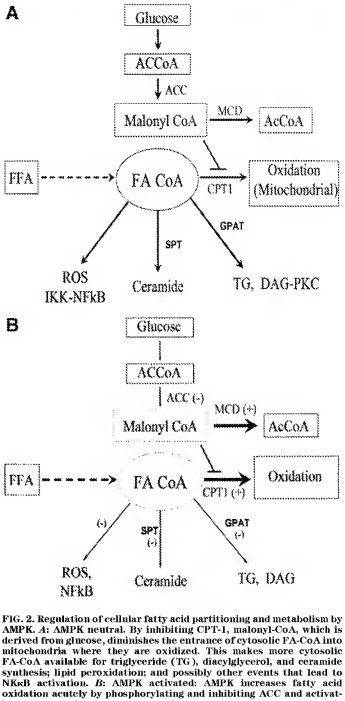

FIG. 2. Regulation of cellular fatty acid partitioning and metabolism by AMPK. *A*: AMPK neutral. By inhibiting CPT-1, malonyl-CoA, which is derived from glucose, diminishes the entrance of cytosolic FA-CoA into mitochondria where they are oxidized. This makes more cytosolic FA-CoA available for triglyceride (TG), diacylglycerol, and ceramide synthesis; lipid peroxidation; and possibly other events that lead to NKκB activation. *B*: AMPK activated: AMPK increases fatty acid oxidation acutely by phosphorylating and inhibiting ACC and activating MCD, leading to a decrease in malonyl-CoA. It also does this subacutely by effects on ACC, MCD, and CPT-1 abundance at the level of transcription. In addition, AMPK inhibits serine palmitoyltransferase, the first committed enzyme in the de novo pathway for ceramide synthesis and glycerophosphate acyltransferase, which plays a similar role in glycerolipid synthesis. The basis for the ability of AMPK to inhibit oxidant stress (ROS generation) and nuclear factor (NF)-κB activation (inflammation) is not known. Whether AMPK activation enhances or inhibits a process or an enzyme in this scheme is denoted by plus and minus signs, respectively (see full text for details). ACC, acetyl-CoA carboxylase; CPT1, carnitine palmitoyltransferase 1; DAG, diacylglycerol; FA CoA, cytosolic long-chain fatty acyl-CoA; FFA, free fatty acid; GPAT, glycerophosphate acyltransferase; MCD, malonyl-CoA decarboxylase; ROS, reactive O_2 species. Adapted from Ruderman and Prentki (17). See text for additional details.

activator SREBP1C, leading to decreases in ACC abundance, and it increases the expression of the transcriptional coactivator peroxisome proliferator–activated

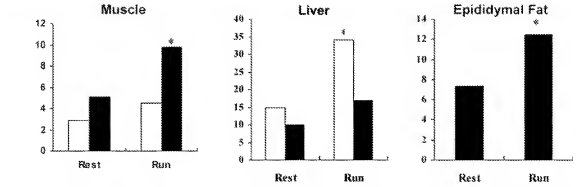

FIG. 3. Effects of 30 min of treadmill running on AMPK activity in muscle, liver, and adipose tissue of normal rats. AMPK activity is expressed in nanomoles per minute per gram protein and was determined in tissue taken from anesthetized rats ~30 min after the run and in control rats that did not run. Activity was determined after immunoprecipitation with α1 (□) and α2 (■) AMPK antibody in muscle and liver and after ammonium sulfate purification in adipose tissue. Reproduced with permission from Park et al. (34).

receptor-γ coactivator 1, PGC1α, and, secondary to this, the transcription factor peroxisome proliferator–activated receptor-α, leading to increases in the synthesis of MCD and CPT-1 itself. Thus, when it is activated, AMPK increases fatty acid oxidation by a number of mechanisms and it does so both acutely and subacutely, underscoring how closely it governs this process.

AMPK also appears to exert multiple controls over the use of cytosolic FA-CoA for other purposes. For instance, by enhancing FA-CoA transfer into mitochondria, it makes less of it available for other processes in the cytosol. In addition, AMPK specifically diminishes the use of cytosolic FA-CoA for the synthesis of diacylglycerol, triglycerides, and phospholipids by decreasing the transcription of glycerophosphate acyltransferase, the first committed enzyme in the glycerolipid synthesis pathway; it diminishes the generation of lipid peroxides and the activation of nuclear factor κB (inflammation) in cells incubated with the fatty acid palmitate (19), and it inhibits the incorporation of palmitate into ceramide, a molecule implicated in causing insulin resistance, oxidative stress, and apoptosis (11,20). Finally, AMPK increases the expression of PGC1α, which in addition to regulating MCD and CPT-1 mRNA, enhances the expression of genes regulating mitochondrial biogenesis and oxidative phosphorylation (21–23), an effect that may be impaired in people with type 2 diabetes and their overweight offspring (21,24,25). It was in large part based on these effects, and the finding that diminished AMPK activity is associated with changes in the opposite direction, that we proposed that dysregulation of AMPK and malonyl-CoA could be pathogenetic factors for the metabolic syndrome as well as targets for its therapy (rev. in 17,26–29).

AMPK ACTIVATION DURING AND AFTER EXERCISE

During exercise, AMPK is activated in contracting skeletal muscle (30,31) in response to increases in the AMP-to-ATP ratio, and it is well established that this contributes to the changes in muscle fuel metabolism that occur during and after physical activity (32,33). Surprisingly, however, in rats studied 30 min after a treadmill run, we also found increases in AMPK activity (Fig. 3) and associated changes in malonyl-CoA content and ACC, MCD, and glycerophosphate acyltransferase activity in liver and adipose tissue (34). Because the energy state of these tissues was not presumably altered, this raised the possibility that AMPK was activated by a systemic hormonal factor.

IL-6: A HORMONE RELEASED FROM SKELETAL MUSCLE DURING EXERCISE

As noted earlier, IL-6 has long been recognized for its effects on the immune system (1), and sustained but modest increases in its plasma concentration have been found in proinflammatory insulin-resistant states. The latter association has been attributed to the presence of obesity, since adipose tissue is perhaps the major contributor to plasma IL-6 in most conditions (4,5). This view of IL-6 has recently been altered by the demonstration of Pedersen and coworkers (35,36) that IL-6 is also a myokine, for which concentration in plasma can rise dramatically during and after exercise as a result of increases in its synthesis and release by contracting skeletal muscle (37). In humans, plasma concentrations of IL-6 may be increased by as much as 100-fold during exercise, to levels far in excess of those observed in obese and diabetic humans (Fig. 4). In general, such increases in IL-6 have paralleled the intensity and duration of exercise, and they were greatest when muscle glycogen levels were low (38). It has been suggested that this increase in IL-6 plays a role in stimulating adipose tissue lipolysis and possibly hepatic glucose production during sustained exercise to provide for the fuel needs of muscle as its endogenous fuel stores (e.g., glycogen) are depleted (35,36). In keeping with this

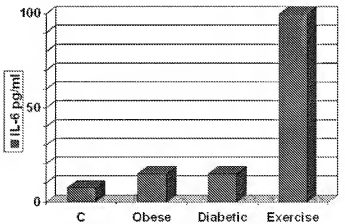

FIG. 4. Plasma levels of IL-6 in obese, type 2 diabetic, and normal control humans and control humans after 1–2 h of moderately intense exercise. Based on data in literature (5).

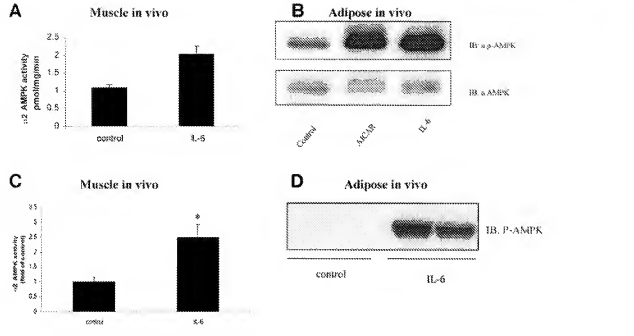

FIG. 5. AMPK activation by IL-6 in (*A*) incubated rat extensor digitorum longus muscle, (*B*) cultured 442A adipocytes, (*C*) rat gastrocnemius muscle, and (*D*) rat epididymal adipose tissue in vivo 1 h after an intraperitoneal injection of IL-6 (2.5 μg). Results are means ± SE (*A*, *C*, and *D*) of four measurements. Blots in *B* are representative of three studies. Adapted from Kelly et al. (41).

notion, IL-6 increases lipolysis upon infusion into humans at rest (39), and it further increases exercise-induced endogenous glucose production (40).

IL-6 ACTIVATES AMPK IN MUSCLE AND ADIPOSE TISSUE

The temporal correlation between changes in plasma IL-6 and tissue AMPK during exercise led us to examine whether the two events are related. As a first test of this notion, we assessed the effect of IL-6 on AMPK activity in rat muscle and F442A adipocytes in vitro. IL-6 markedly increased AMPK in both tissues, with the increase in phospho-AMPK (P-AMPK) abundance (an index of activity) in the fat cells comparable to that produced by the classic AMPK activator 5-aminoimidazole-4-carboxamide riboside (AICAR) (Fig. 5). We have found similar IL-6–induced increases in AMPK in incubated pancreatic islets (A.-M.R., M.K., unpublished data) and in rat muscle and adipose tissue after an intraperitoneal injection of IL-6 (Fig. 5). Also of note, the increase in AMPK activity in incubated muscle was transient, and values below those of control muscles were observed by 60 min (41). Whether this secondary decrease in AMPK activity persists during longer periods of incubation with IL-6 and whether a similar pattern occurs in vivo and in other tissues remains to be determined.

STUDIES IN THE IL-6 KO MOUSE

To assess more directly whether IL-6 causes the increase in tissue AMPK during and after exercise, we compared AMPK activity in muscle, adipose tissue, and liver of control mice and IL-6 knockout (IL-6 KO) mice after 1 h of swimming (41). In keeping with previous studies in the rat (34), exercise increased the abundance of both P-AMPK and phospho-ACC (P-ACC) in muscle, liver, and adipose tissue of the control mice. No obvious difference in the ability of the control and IL-6 KO mice to perform exercise was observed. Despite this, in the IL-6 KO mice at rest, the abundance of P-AMPK and P-ACC was diminished by 75–90% in muscle (Fig. 6) and to a somewhat lesser extent in adipose tissue. After exercise, similar percentage increases in P-AMPK and P-ACC abundance were observed in muscle and adipose tissue of the control and IL-6 KO mice; however, because of their lower initial values, the absolute levels of P-AMPK and P-ACC achieved were lower in the IL-6 KO mice. For reasons unknown, P-AMPK and P-ACC were only minimally diminished in liver of the mice lacking IL-6, and the increments in their abundance produced by exercise were indistinguishable from those of control mice. Collectively, these data strongly suggest that IL-6 is a significant regulator of the basal activity of AMPK in muscle and adipose tissue. They also suggest that it is a key factor regulating the increase in tissue P-AMPK and P-ACC caused by exercise, but not the only factor. As discussed elsewhere (41), another likely regulator of the effects of exercise on AMPK is the sympathetic nervous system, which itself may be activated in part by a central action of IL-6 (42). Perhaps relevant to this discussion, it has recently been observed that transgenic mice overexpressing IL-6 do not develop obesity and insulin resistance as do control mice when fed a high-fat diet (M.F. White, personal communication).

IL-6 KO MOUSE AS A MODEL OF THE METABOLIC SYNDROME

At 3 months of age, the IL-6 KO mouse is not obese, whereas by 9 months of age, it is obese, hypertriglyceridemic, and glucose intolerant (42), which by definition gives it a diagnosis of the metabolic syndrome (43). Decreases in AMPK activity have been observed in a number of rodents that either have or subsequently develop manifestations of the metabolic syndrome. As listed

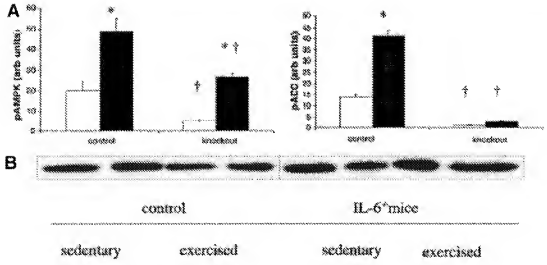

FIG. 6. AMPK and ACC phosphorylation and AMPK abundance in gastrocnemius muscle of control and IL-6 KO mice and the effect of exercise (1 h of swimming). *A*: **P-AMPK and P-ACC abundance in sedentary (□) and exercised (■) control and IL-6 KO (n = 4) mice. Results are means ± SE. *P < 0.05 vs. resting value; †P < 0.01 vs. control value.** *B*: **Abundance of total AMPK (α1- and α2-subunit) protein in control and IL-6 KO mice at rest and after exercise. Results are representative of four blots in each condition. Similar findings after exercise were observed in adipose tissue but not in liver. Adapted from Kelly et al. (41).**

in Table 1, they include rodents that are obese and not obese, diabetic and nondiabetic, and hypertriglyceridemic and normoglyceridemic, with insulin resistance a common denominator. In one of these rodents, the glucose-infused rat, insulin resistance, ectopic lipid accumulation, and decreased AMPK activity in liver all first appeared between 3 and 5 h, suggesting they are early events (44). AMPK activation has been demonstrated to diminish insulin resistance and other abnormalities in a number of these animals, suggesting it plays a causal role. For instance, treatment of the ZDF rat with AICAR (45,46) or regular exercise (46) has been shown to prevent the development of diabetes, as has treatment with thiazolidinedione therapy and calorie restriction (47), both of which have also been reported to activate AMPK in rats (48,49). Similar benefits of exercise (50) and AICAR (51) have been reported in rats made insulin resistant by fat feeding.

Several characteristics of the IL-6 KO mouse suggest that the early decrease in AMPK activity in muscle and adipose tissue of these mice could contribute to the metabolic syndrome phenotype observed at 9 months. Thus, Faldt et al. (52), based on measurements of respiratory exchange ratio at 3 months of age (respiratory exchange ratio 0.92 vs. 0.82 in control mice), concluded that the ability of the IL-6 mouse to oxidize fatty acids is impaired. Interestingly, they also noted that by 9 months of age, the respiratory exchange ratio of these mice had decreased to 0.82 (same as control mice). Whether this reflected an increased availability of fatty acids as a result of obesity and/or a restoration of AMPK activity remains to be determined. Also of note, Faldt et al. (52) found that the ability of both 3- and 9-month-old IL-6 KO mice to sustain exercise was diminished, suggesting impaired cardiovascular, pulmonary, or muscle function. In keeping with the latter possibility in a very preliminary study (in 3-month-old IL-6 KO mice), we found decreased levels of cardiolipin, a mitochondrial lipid whose concentration diminishes when mitochondria are damaged (e.g., as seen when apoptosis and impaired mitochondrial function are caused by incubation of cells with the fatty acid palmitate) (53) (Table 2). In addition, we found a reduced (50%) abundance in muscle of the mRNA of mitochondrial uncoupling protein (UCP)-3. Diminished size and efficiency of mitochondria have been observed in muscle of patients with type 2 diabetes (54). Whether decreased AMPK activity, by virtue of its effects on cellular lipid metabolism, mitochondrial genes, or other factors, causes these abnormalities to our knowledge has not been studied. On the other hand, it has been demonstrated that AMPK activation prevents both the apoptosis and mitochondrial dysfunction observed in cultured human umbilical vein endothelial cells incubated in a high-glucose medium (55) and the apoptosis, inflam-

TABLE 1
Characteristics of rodents in which decreased AMPK activity precedes or is associated with aspects of the metabolic syndrome (see text for details)

	Obese	Insulin resistant	Ectopic lipid	Dyslipidemia	Hyperglycemia
fa/fa rat	++	+	+	+	−
ZDF rat	++	+	+	+	++
Fat-fed rat	++	+	+	+	+
Glucose-infused rat	−	+	+	ND	+
Dahl-S rat	−	+/−	−	+	−
IL-6 KO mouse					
3 months	−	ND	ND	−	−
9 months	+	ND	ND	+	+

ND, no data.

TABLE 2
Decreased cardiolipin and UCP3 mRNA in white, but not red, gastrocnemius muscle of 3-month-old IL-6 KO mice

	Cardiolipin (μg/mU creatine kinase)		NADH oxidase (units/mU creatine kinase)		UCP3 content (arbitrary units)
	White	Red	White	Red	White
Control	114	134	0.75	1.24	0.72 ± 0.05
	118	130	0.68	1.41	
IL-6 KO	67	156	0.63	1.48	0.373 ± 0.05*
	77	180	0.78	1.55	

Similar changes were not observed for the NADH oxidase. In keeping with previous reports, NADH oxidase activity was higher in red muscle, which is richer in mitochondria. Cardiolipin and NADH oxidase results are for muscles from two mice. UCP3 data are means ± SE (n = 7–10). *P < 0.005. UCP3, uncoupling protein 3. See text for additional details.

mation, and oxidative stress observed when human umbilical vein endothelial cells are incubated with a modestly elevated concentration of palmitate (19).

IL-6 AND THE METABOLIC SYNDROME: A CONUNDRUM

In addition to being predisposed to obesity, dyslipidemia, and glucose intolerance, IL-6 KO mice demonstrate more advanced atherosclerosis when bred on an Apo $E^{-/-}$ background than do Apo $E^{-/-}$ mice in which IL-6 is not lacking (56,57). On the other hand, humans with modest elevations of IL-6, attributable to obesity (5), are also predisposed to diabetes and atherosclerotic vascular disease (58). It is tempting to speculate that differences in AMPK activity in response to IL-6 in various tissues could contribute to this paradox; however, there is as yet no evidence for or against this notion. In this context, studies of both the mechanism by which AMPK is activated by IL-6 and the effects of AMPK activation on different components of the IL-6 signaling pathway could prove interesting (Fig. 7).

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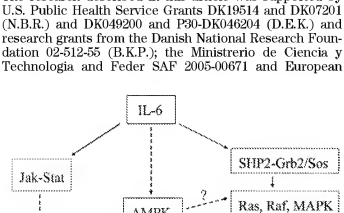

FIG. 7. Hypothetical scheme to explain how AMPK activation by IL-6 could influence its actions on the cell. AMPK is known to inhibit NFκB activation and JNK, mTOR, and other cellular enzymes by multiple means (see Fig. 2 and accompanying text). Whether it selectively exerts an effect on some of the signals or genes altered by IL-6 is presently under study.

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Palmitate activates AMP-activated protein kinase and regulates insulin secretion from beta cells.

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AMP-activated protein kinase (AMPK) is an energy sensor that regulates cellular metabolism. Changes in AMPK activity contribute to the regulation of insulin secretion. Epidemiological evidence links the ingestion of saturated fatty acid with hyperinsulinemia. The aim of the present study was to examine the effects of palmitate on beta cell AMPK activity and insulin secretion. Isolated rat islets and MIN6 beta cells were treated acutely (5-60 min) or chronically (24 h) with palmitate. Insulin secretion, AMPK and acetyl CoA carboxylase phosphorylation were assessed. The acute effects of palmitate included AMPK activation and augmentation in insulin secretion. Activation of AMPK by 24h pretreatment with palmitate suppressed glucose-stimulated insulin secretion, but not the response of insulin secretion to combined stimuli of glucose and palmitate. This study demonstrated that palmitate availability affected beta cell AMPK activity. In beta cells, an increase in AMPK activity may be required for fatty acid-induced fatty acid oxidation and prevention of lipotoxicity.

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Statins Activate AMP-Activated Protein Kinase In Vitro and In Vivo

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Background—Statins exert pleiotropic effects on the cardiovascular system, in part through an increase in nitric oxide (NO) bioavailability. AMP-activated protein kinase (AMPK) plays a central role in controlling energy and metabolism homeostasis in various organs. We therefore studied whether statins can activate AMPK, and if so, whether the activated AMPK regulates nitric oxide (NO) production and angiogenesis mediated by endothelial NO synthase, a substrate of AMPK in vascular endothelial cells.

Methods and Results—Western blotting of protein extracts from human umbilical vein endothelial cells treated with atorvastatin revealed increased phosphorylation of AMPK at Thr-172 in a time- and dose-dependent manner. The AMPK activity, assessed by SAMS assay, was also increased accordingly. The phosphorylation of acetyl-CoA carboxylase at Ser-79 and of endothelial NO synthase at Ser-1177, 2 putative downstream targets of AMPK, was inhibited by an adenovirus that expressed a dominant-negative mutant of AMPK (Ad-AMPK-DN) and compound C, an AMPK antagonist. The positive effects of atorvastatin, including NO production, cGMP accumulation, and in vitro angiogenesis in Matrigel, were all blocked by Ad-AMPK-DN. Mice given atorvastatin through gastric gavage showed increased AMPK, acetyl-CoA carboxylase, and endothelial NO synthase phosphorylation in mouse aorta and myocardium.

Conclusions—Statins can rapidly activate AMPK via increased Thr-172 phosphorylation in vitro and in vivo. Such phosphorylation results in endothelial NO synthase activation, which provides a novel explanation for the pleiotropic effects of statins that benefit the cardiovascular system. (***Circulation*. 2006;114:2655-2662.**)

Key Words: statins ■ angiogenesis ■ aorta ■ endothelium ■ nitric oxide ■ nitric oxide synthase ■ myocardium

The clinical efficacy of statin therapy in decreasing cardiovascular mortality and morbidity is clearly demonstrated by several cohort trials, such as the Heart Protection Study.[1] Functioning as competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, statins increase the hepatic expression of the low-density lipoprotein receptor, which results in enhanced low-density lipoprotein clearance in the circulation. In addition to their cholesterol-lowering effect, statins display other cardiovascular protective effects. Such pleiotropic effects on the vessel wall have been suggested to contribute to antioxidative, antiinflammatory, and improved endothelial functions through enhanced bioavailability of nitric oxide (NO; see Wolfrum et al[2] for review). In the experimental myocardial infarction model in mice, atorvastatin treatment was shown to enhance neovascularization in myocardium.[3] Simvastatin administration also promoted angiogenesis in ischemic limbs of normocholesterolemic rabbits.[4] At the cellular and molecular levels, treatment of vascular endothelial cells (ECs) with statins activates endothelial NO synthase (eNOS), with increased NO production, which has been suggested to be mediated through the phosphatidylinositol-3 kinase–Akt/PKB pathway.[5]

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AMP-activated protein kinase (AMPK) is a trimeric enzyme comprising a catalytic α-subunit and regulatory β,γ-subunits.[6] AMPK was first identified as an upstream kinase that phosphorylates and hence inactivates 3-hydroxy-3-methylglutaryl coenzyme A reductase and acetyl-CoA carboxylase (ACC), the key enzymes controlling cholesterol/isoprenoid and fatty acid biosynthesis, respectively. AMPK can function as a fuel gauge to regulate the homeostasis of energy in the form of glucose and fatty acids in skeletal muscles, liver, and adipocytes (see Kahn et al[7] and Rutter et

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al[8] for review). Recent findings suggest that the fuel-sensing mechanism of AMPK is also present in the hypothalamus to regulate food intake, energy expenditure, and body weight.[9,10] The involvement of AMPK in diabetes mellitus is demonstrated by insulin resistance, with associated high levels of plasma glucose and low levels of insulin in mice with ablated AMPK-α2.[11]

Although Akt has been demonstrated to be a major kinase phosphorylating human eNOS at Ser-1177 (Ser-1179 in bovine eNOS), with ensuing increased activity of eNOS,[12] AMPK can also phosphorylate eNOS at Ser-1177/1179, particularly in ECs.[13] AMPK may have a beneficial effect on the vessel wall, because several recent studies demonstrated that adiponectin, high-density lipoprotein, apolipoprotein AI, estradiol, and shear stress all activate AMPK in ECs, with augmented NO production.[13–18] Given the pleiotropic effects of statins on endothelium-mediated vascular functions, we investigated the role of AMPK in statin-induced eNOS phosphorylation and NO bioavailability in vitro and in vivo. Our results demonstrate for the first time that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors activate AMPK in ECs. At a clinical dosage, atorvastatin activates AMPK in the vessel wall and the myocardium in mice, with attendant activation of eNOS.

Methods

Materials

Antibodies used in the present study and their commercial sources were as follows: anti-phospho-AMPK Thr-172, anti-pan-α-AMPK, anti-phospho-Akt Ser-473, and anti-phospho-ACC Ser-79 (Cell Signaling Technology, Beverly, Mass); anti-phospho-eNOS Ser-1177/1179, polyclonal anti-eNOS (BD Biosciences Pharmingen, San Diego, Calif); and anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz, Calif). Griess reagent, 5-aminoimidazole-4-carboxamide 1-ribofuranoside (AICAR), and 2,4-dinitrophenol were purchased from Sigma (St. Louis, Mo), whereas compound C was from Calbiochem (San Diego, Calif). Matrigel was obtained from BD Biosciences (San Jose, Calif). Atorvastatin and lovastatin were from Toronto Research Chemicals, Inc (North York, Canada) and A.G. Scientific (San Diego, Calif), respectively. The specific AMPK-targeted SAMS peptide used in AMPK activity assays was from GenScript (Piscataway, NJ).

Cell Culture, Adenovirus, and EC Infection

Human umbilical vein endothelial cells (HUVECs) were cultured in medium M199 (Gibco Life Technology, Karlsruhe, Germany) with 15% fetal bovine serum (Omega, Tarzana, Calif), 3 ng/mL β-endothelial cell growth factor (Sigma), 4 U/mL heparin, and 100 U/mL penicillin-streptomycin. Human capillary ECs obtained from Clonetics (San Diego, Calif) and bovine aortic ECs isolated from bovine aorta were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, Calif) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. ECs were maintained in a humidified 95% air–5% CO_2 incubator at 37°C. Cells within passages 2 to 5 were used in all experiments. We used a recombinant adenovirus expressing a dominant-negative mutant of AMPKα1, henceforth referred to as Ad-AMPK-DN, described previously.[18,19] The parental adenoviral vector was referred to as null when used as a control. Confluent ECs were infected with recombinant adenoviruses at the indicated multiplicity of infection and incubated for 24 hours before experiments.

Detection of NO and cGMP Assay

We determined accumulated nitrite (NO_2^-), a stable breakdown product of NO, in culture media by mixing an aliquot of cell culture media with an equal volume of Griess reagent and then incubating it at room temperature for 15 minutes. The azo dye production was analyzed by use of a spectrophotometer with absorbance set at 540 nm. Sodium nitrite was used as a standard. Intracellular levels of cGMP in ECs were assessed over 4 hours. After removal of culture media, ECs were lysed, and the extracts were collected and centrifuged for 5 minutes at 5000g. cGMP level was determined by use of a cGMP enzyme immunoassay kit (R&D Systems, Minneapolis, Minn), then normalized to protein content as determined by Bradford assay.

Western Blotting

ECs were lysed with a buffer that contained 10 mmol/L Tris, pH 7.4, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L NaF, 20 mmol/L $Na_4P_2O_7$, 2 mmol/L Na_3VO_4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X 100, 10% glycerol, 10 μg/mL leupeptin, 60 μg/mL aprotinin, and 1 mmol/L phenylmethanesulfonyl fluoride. Frozen mouse aortas and hearts were thawed and homogenized in the same buffer as above. EC lysates and mouse aortic and myocardial extracts were resolved on SDS-PAGE according to standard protocols. After being transferred to membranes, the samples were immunoblotted with primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase. Bands were revealed by use of an enzyme-linked chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ), and density was quantified by use of Scion Image software (Scion Corp, Frederick, Md).

In Vitro Angiogenesis (Tube Formation) Assays

The angiogenic effect of the statin-activated AMPK was examined with the use of human capillary ECs. Cells were infected with recombinant adenovirus for 24 hours, then seeded on Matrigel (3×10^4 cells/cm^2) in 24-well culture plates for another 24 hours, in the presence or absence of atorvastatin. Tube formation was assessed by microscopic imaging and quantified by counting the number of branch points.

AMPK Activity Assay and High-Performance Liquid Chromatography Determination of Cellular AMP and ATP

EC lysates were incubated with SAMS peptide and (γ-^{32}P)ATP, and the catalytic activity of AMPK was determined by the incorporation of ^{32}P into SAMS peptide. HUVECs were treated with atorvastatin or 2,4-dinitrophenol, and nucleotides were extracted according to published procedures.[20] The high-performance liquid chromatography used was composed of a Surveyor MS pump, a Surveyor PDA (Thermo, Waltham, Mass), and a YMC ODS-AQ S-5 column (4.6×250 mm, 5 μm in particle size, and 120 Å in pore size; YMC Co, Ltd, Kyoto, Japan). The flow rate was 500 μL/min, and a UV detector was set at 260 nm to monitor the fractions. The mobile phases were 50 mmol/L triethylammonium acetate, pH 6.5 (buffer A) and 30% acetonitrile in A (buffer B), and the gradient program was composed of 40-minute 0% to 90% buffer B, 5-minute 90% to 100% buffer B, and 1-minute 100% to 0% buffer B.

Animal Experiments

The animal experimental protocols were approved by the UCR institutional Animal Care and Use Committee. All experiments were performed in 8–week-old male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Me). Mice were fed with atorvastatin at 50 mg/kg body weight by gastric gavage. Saline was fed to control animals as a vehicle control. After 2, 4, 8, 12, or 24 hours, mice were killed. Abdominal aortas and hearts were removed and stored in −80°C.

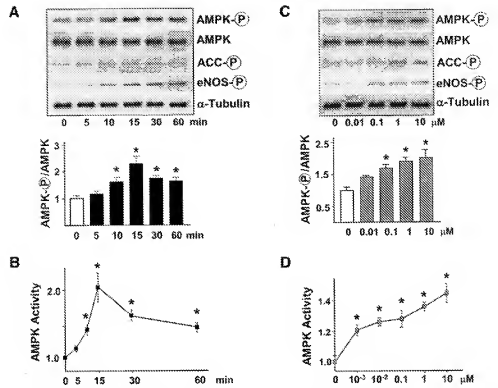

Figure 1. Atorvastatin activates AMPK in cultured ECs. HUVECs were treated with (A) atorvastatin 1 μmol/L for the times indicated and (C), various concentrations of atorvastatin for 10 minutes. The cells were then lysed, and 100 μg of the cell lysates underwent SDS-PAGE followed by Western blotting with various primary antibodies as shown, then were visualized by the enzyme-linked chemiluminescence system. The bar graphs below are densitometry analyses of the ratio of phosphorylated AMPK to that of total AMPK. Data presented are mean±SD from 5 independent experiments, with nontreated controls set as 1. In separate sets of experiments shown in B and D, 2 μg of protein extracts underwent AMPK activity assays with SAMS peptide and (γ-^{32}P)ATP used as substrates. The data represent mean±SD from 3 separate experiments. *P<0.05 between atorvastatin-treated groups and nontreated controls.

Statistical Analysis

The significance of variability was determined by unpaired Student t test or ANOVA. Each experiment included triplicate measurements for each condition tested, unless otherwise indicated. All results are expressed as mean±SD from at least 3 independent experiments. In all cases, P<0.05 was considered to be statistically significant.

The authors had full access to the data and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Statins Increase AMPK, ACC, and eNOS Phosphorylation in Cultured ECs

To test whether statins can phosphorylate AMPK in ECs, HUVECs were treated with atorvastatin for up to 60 minutes. As shown in Figure 1A, the phosphorylation of Thr-172 of AMPK in HUVECs increased transiently, with a peak level at 15 minutes. Treatment with atorvastatin did not change the levels of AMPK in ECs. The transient increase in AMPK phosphorylation was accompanied by transient augmentation of phosphorylation of ACC at Ser-79, a downstream target of AMPK. As a putative effector of AMPK, eNOS was also phosphorylated at Ser-1177 in HUVECs in response to atorvastatin. The increased AMPK phosphorylation coincided with an augmented AMPK activity, as assessed by SAMS peptide assay (Figure 1B). In control cells, the addition of MeOH (atorvastatin vehicle) with a dilution of 1:10^4 (vol/vol) did not increase any of these phosphorylation events (data not shown). As shown in Figure 1C and 1D, AMPK, ACC, and eNOS phosphorylation and AMPK activity increased in an atorvastatin dose–dependent manner. The activation of AMPK by the statin was also observed in bovine aortic ECs stimulated with lovastatin (online-only Data Supplement, Figure I).

Statin-Activated AMPK Is Involved in eNOS Phosphorylation and NO Production

We have previously demonstrated that ECs infected with a recombinant adenovirus expressing the constitutively active form of AMPK (Ad-AMPK-CA) showed activated eNOS and increased NO production.[18] Given that statins activate both AMPK and eNOS in ECs in a time- and dose-dependent manner, we then investigated whether the statin-activated eNOS and the consequent NO production involves AMPK. HUVECs infected with Ad-AMPK-DN were incubated with atorvastatin 1 μmol/L for 10 minutes. As shown in Figure 2A, cells infected with the Ad-null control showed an increased phosphorylation of AMPK at Thr-172, ACC at Ser-79, and eNOS at Ser-1177. However, Ad-AMPK-DN expression, as indicated by positive antihemagglutinin Western blotting, attenuated these phosphorylation events. Functional assays shown in Figure 3A and 3B demonstrated that treatment with atorvastatin enhanced the NO production and

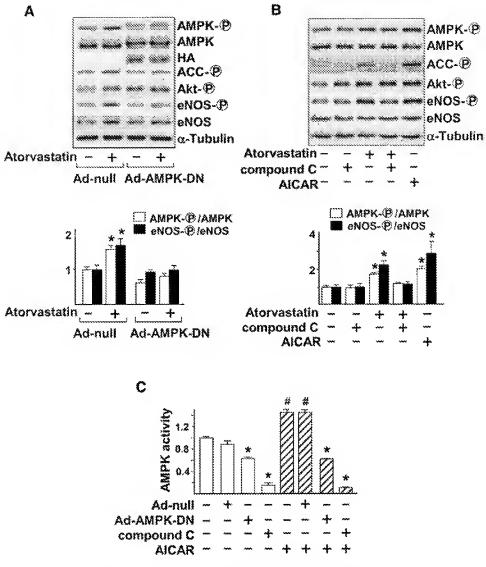

Figure 2. AMPK mediates statin-phosphorylated ACC and eNOS in ECs. A, HUVECs were infected with control null virus (Ad-null, 50 multiplicities of infection) or an adenovirus expressing the dominant-negative mutant of AMPK (Ad-AMPK-DN, 50 multiplicities of infection) for 24 hours. The infected cells were then treated with atorvastatin (1 μmol/L) for 10 minutes. B, HUVECs were treated with compound C (20 μmol/L) for 20 minutes or were untreated before atorvastatin (1 μmol/L) treatment for 10 minutes. In parallel positive controls, cells were treated with AICAR (1 mmol/L) for 20 minutes. The phosphorylation of AMPK, ACC, and eNOS was analyzed by Western blotting. The bands revealed by anti-hemagglutinin (HA) in A indicate the expression of the exogenous hemagglutinin-AMPK-DN. C, The activity of AMPK in ECs with various conditions as indicated was measured by SAMS assays. The data represent results of 4 separate experiments. In A, $^*P<0.05$ between atorvastatin-treated and nontreated cells infected with Ad-null. In B, $^*P<0.05$ between atorvastatin- or AICAR-treated cells and nontreated controls. In C, $^*P<0.05$ between Ad-AMPK-DN or compound C groups and nontreated controls, and $\#P<0.05$ between AICAR-treated groups and nontreated controls.

intracellular cGMP accumulation, which was attenuated in cells expressing AMPK-DN. Interestingly, atorvastatin and AICAR, an AMPK agonist, had similar effects in augmenting the phosphorylation of ACC and eNOS in HUVECs (Figure 2B). Such atorvastatin-increased phosphorylation of ACC and eNOS was also abolished by compound C, an AMPK antagonist (Figure 2B). The AMPK activity in both the Ad-AMPK-DN–infected and compound C–treated cells was reduced to a level lower than that of control cells (Figure 2C). However, the lack of AMPK activity by either Ad-AMPK-DN or compound C increased Akt Ser-473 phosphorylation regardless of the presence or absence of atorvastatin, as shown in Figure 2A and 2B.

AMPK Mediates Statin-Induced Tube Formation

Statins have been shown to facilitate EC-derived angiogenesis in vitro and in vivo, which is mediated through the eNOS-produced NO.[4] To investigate further the role of AMPK in statin-enhanced NO bioavailability, we performed tube formation assays. Human capillary ECs infected with null adenovirus or Ad-AMPK-DN were seeded on Matrigel in the presence or absence of atorvastatin. As shown in Figure 4, atorvastatin enhanced EC tube formation in Matrigel regardless of the presence of the Ad-null. However, the atorvastatin-induced tube formation was significantly attenuated with Ad-AMPK-DN infection or compound C treatment. These results agree with the notion that AMPK is involved in

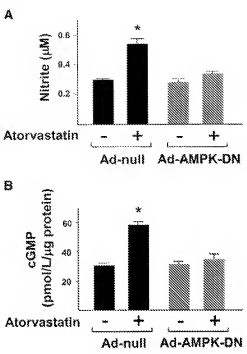

Figure 3. AMPK is involved in statin-enhanced NO production in ECs. HUVECs were infected with Ad-null or Ad-AMPK-DN (50 multiplicities of infection). The infected ECs were then treated with atorvastatin (1 μmol/L) for 4 hours. NO production in ECs was revealed by Griess assay (A) and accumulation of intracellular cGMP (B). *$P<0.05$ between atorvastatin-treated and non-treated ECs.

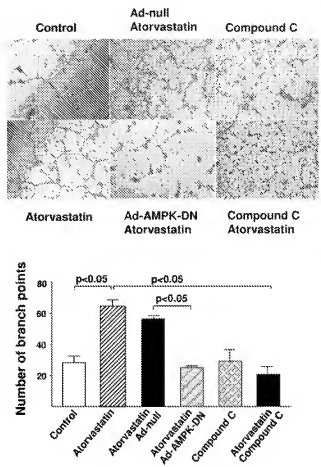

Figure 4. AMPK is involved in atorvastatin-induced angiogenesis in vitro. Human capillary ECs cultured in Matrigel were kept as controls or treated with atorvastatin (1 μmol/L) for 24 hours. In parallel sets of experiments, human capillary ECs were infected with Ad-null (50 multiplicities of infection) or Ad-AMPK-DN (50 multiplicities of infection) or treated with compound C (10 μmol/L), in the presence or absence of atorvastatin. The photos are representative micrographs (magnification ×40) of the indicated experiments. The bar graphs below indicate the number of branch points in the full microscopy view (mean±SD) averaged from results of 3 separate experiments.

statin-enhanced angiogenesis because of increased NO bioavailability.

Atorvastatin Activates AMPK Phosphorylation In Vivo

To explore whether AMPK in the vessel wall and heart can be activated by statin in vivo, C57BL/6J mice were given atorvastatin at 50 mg/kg body weight, then aorta and apex myocardium were removed at different time points (2 to 24 hours) to detect the level of AMPK, ACC, and eNOS phosphorylation. As shown in Figure 5A and 5B, the level of phosphorylated AMPK Thr-172 in aorta and myocardium increased 2 to 4 hours after atorvastatin administration and lasted for at least 24 hours. The phosphorylation of ACC and eNOS was elevated, with a similar pattern to that of phosphorylated AMPK. The activity of AMPK increased in these tissues as well, as revealed by SAMS assay (Figure 5C and 5D).

Discussion

Results from the Scandinavian Simvastatin Survival Study (4S) and the Heart Protection Study demonstrate clearly that statin therapy achieves a greater reduction of both myocardial infarction and death rate.[1,21] Although statins reduce cardiovascular incidents, anti-type II diabetic drugs such as metformin and rosiglitazone, a peroxisome proliferator–activated receptor-γ agonist, can activate AMPK.[20,22] Although AMPK has emerged as a new target for treating metabolic syndromes in various tissue types, the principal finding of the present study is that statins also activate AMPK in the cardiovascular system.

We used atorvastatin to demonstrate the positive effect of statins on the phosphorylation of AMPK Thr-172 in ECs. Such phosphorylation is essential for AMPK activation, which is also revealed by increased AMPK activity and increased phosphorylation of ACC Ser-79, a direct target of AMPK.[23,24] The positive effect of statins should not be limited to atorvastatin, because lovastatin can also cause AMPK and ACC phosphorylation in bovine aortic ECs (online Data Supplement, Figure I). A previous study by Xenos et al[25] showed an increase in the level of AMPK protein in human ECs treated with fluvastatin for 48 hours. The present data indicate that AMPK phosphorylation/activity in ECs was increased by statins in a rapid and transient manner. This temporal response is similar to that stimulated by shear stress, peroxisome proliferator–activated receptor-γ agonists, adiponectin, metformin, estradiol, and high-density

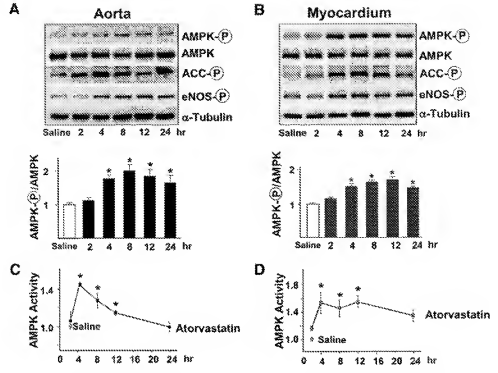

Figure 5. Atorvastatin activates AMPK in mouse models in vivo. C57BL/6 mice were killed after atorvastatin treatment (50 mg/kg) for the indicated times. In the control group, mice received the same amount (0.5 mL) of saline 2 hours before they were killed. Tissue extracts from aorta (A) and heart (B) were analyzed by Western blotting with various antibodies as indicated. The data represent results of 6 independent sets of experiments. The bottom panels of A and B are densitometry quantification revealing the ratio between levels of phosphorylated AMPK normalized to those of total AMPK. AMPK kinase activity assays with the use of SAMS peptide as a substrate were also performed for extracts from aorta (C) and myocardium (D). Results shown are mean±SD averaged from 4 animals for each time point. *$P<0.05$ between saline-treated controls and atorvastatin-treated groups.

lipoprotein.[13,16–18,26] Rosiglitazone activates AMPK by increasing the cellular AMP/ATP ratio.[20] With examination by high-performance liquid chromatography, atorvastatin treatment did not alter the AMP/ATP ratio (online Data Supplement, Figure II), which is similar to the effect of metformin treatment.[20] The phosphorylation/activation of AMPK in mouse aorta and myocardium was observed as early as 2 hours and peaked at 4 to 8 hours after atorvastatin administration (Figure 5). The delay of atorvastatin delivery to circulation due to gastric administration in these mice may account for the discrepant temporal responses of AMPK in vitro in cultured cells and in vivo in mice. Notably, cardiomyocytes constitute the major cell types in the heart, and atorvastatin also caused increased AMPK and ACC phosphorylation in cardiomyocytes (W.S. and J.Y.-J.S., unpublished data). Thus, the tissue sources of the detected AMPK activation in the myocardium in vivo would be both endothelium and cardiomyocytes.

The effect of statins on eNOS activation and the resultant NO release in ECs have been documented to be dependent on the phosphatidylinositol-3 kinase–Akt pathway.[4,12] The present results indicate that AMPK is also engaged in the upregulation of eNOS-NO by statins. The experimental evidence supporting such an argument is the inhibition of eNOS Ser-1177 phosphorylation, NO/cGMP production, and tube formation by Ad-AMPK-DN and compound C in vitro. The association between Akt and AMPK in phosphorylating eNOS Ser-1177/1179 is elusive (see Sessa[27] for review). Wortmannin, a phosphatidylinositol-3 kinase inhibitor, could block the phosphorylation of eNOS Ser-1177/1179 in ECs in response to shear stress, which indicates that eNOS is phosphorylated by phosphatidylinositol-3 kinase–Akt.[12] However, later studies showed that dominant-negative mutants of Akt were unable to inhibit eNOS phosphorylation, although these mutants could inhibit shear-dependent NO release.[28,29] Results of the present immunoprecipitation kinase activity assays revealed that AMPK immunoprecipitated from sheared ECs phosphorylated glutathione *S*-transferase–eNOS, which indicates that AMPK can phosphorylate eNOS directly.[18] This result agrees with those by Chen et al[13] showing that a dominant-negative mutant of AMPK but not of Akt significantly inhibited eNOS phosphorylation and NO production in ECs in response to adiponectin. Although eNOS Ser-1177 phosphorylation was largely inhibited in the Ad-AMPK-DN–infected or compound C–treated HUVECs, Akt Ser-473 phosphorylation was drastically increased in these cells that lacked AMPK activity (Figure 2). The increased Akt phosphorylation may be due to the loss of feedback inhibition of insulin receptor signaling that has been observed with ablated AMPK.[30,31] Data presented in Figure 2 support the hypothesis that eNOS activation does not depend on Akt, because inhibition of AMPK mitigated the statin-activated eNOS despite increased Akt phosphorylation. However, others have shown that the dominant-negative mutant of

Akt blocked adiponectin-stimulated Akt and eNOS phosphorylation without altering AMPK phosphorylation, but dominant-negative AMPK inhibited adiponectin-induced Akt phosphorylation.[26] These results suggest that AMPK is upstream of Akt. Regardless of the hierarchy or parallel role of AMPK in activating eNOS-NO in relation to Akt, data presented in Figure 4 demonstrate that statin-induced angiogenesis, a crucial indication of endothelial NO bioavailability, is mediated at least in part through AMPK.

A dose of 50 mg of atorvastatin per kilogram of body weight in mice corresponds to ≈80 mg/d in humans.[3,32] The rapid activation of AMPK-eNOS/ACC in the aorta and myocardium by this therapeutic dose may have clinical implications. Endothelium has been considered an organ system that is not meant for fatty acid biosynthesis and storage. Fatty acids have been suggested to be a major energy source of ECs,[33] however, and thus the effect of the statin-activated ACC in ECs remains to be investigated. In the ischemic heart, the oxidation of both free fatty acids and glucose is inhibited, but glucose transport and ATP production resulting from glycolysis are increased (see Young et al[34] for review). Apparently, AMPK in cardiomyocytes plays a central role in these metabolic regulations. Transgenic mice expressing a kinase-dead mutant of AMPK showed increased apoptosis and cardiac dysfunction after ischemia-reperfusion injury ex vivo.[35] Furthermore, adiponectin has been shown to protect the heart from ischemia-reperfusion injury, which is AMPK dependent.[36] Thus, during ischemic heart disease, statin-activated AMPK may be beneficial not only through improved coronary endothelial function and myocardial neovascularization but also through exertion of a cardioprotective effect. Given that metformin, thiazolidinediones, and adiponectin can activate AMPK in various metabolically related organs, including skeletal muscle, liver, and pancreatic islets,[23,37–40] statin-activated AMPK is also likely to be present in tissues other than cardiovascular cells. If so, the advantages of AMPK activation in response to statins may extend beyond the protection of vascular endothelium and myocardium.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The efficacy of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in preventing and treating cardiovascular diseases is supported by the results of many evidence-based clinical studies. In addition to cholesterol lowering, the benefits of statins include pleiotropic effects on the cardiovascular system. Because multiple mechanisms are involved in the efficacious effects of statins, we explored the connection between statins and AMP-activated protein kinase (AMPK), a protein kinase that modulates metabolic homeostasis and energy balance in individual cell and in multiple organs. We demonstrate for the first time that atorvastatin, one of the most widely prescribed statins, can rapidly activate AMPK in cultured endothelial cells. At a clinical dosage, atorvastatin also activates AMPK in the mouse aorta and myocardium, with attendant endothelial nitric oxide synthase activation. The activated endothelial nitric oxide synthase in turn increases nitric oxide production and enhances endothelial cell–mediated angiogenesis. Such an augmentation of nitric oxide bioavailability may enhance the functional modulation of blood vessels, increase blood and oxygen supply, and promote revascularization after the onset of ischemic myocardial injury. Our finding provides a novel explanation for the pleiotropic effects of statins on the cardiovascular system. Because AMPK has emerged as a new target for treating metabolic syndromes in various tissue types, our results suggest that statins, as a modulator of AMPK, may help maintain metabolic homeostasis and energy balance in metabolic syndromes.

Inhibition of lipid synthesis through activation of AMP kinase: an additional mechanism for the hypolipidemic effects of berberine

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Abstract The alkaloid drug berberine (BBR) was recently described to decrease plasma cholesterol and triglycerides (TGs) in hypercholesterolemic patients by increasing expression of the hepatic low density lipoprotein receptor (LDLR). Using HepG2 human hepatoma cells, we found that BBR inhibits cholesterol and TG synthesis in a similar manner to the AMP-activated protein kinase (AMPK) activator 5-aminoimidazole-4-carboxamide 1-β-ribofuranoside (AICAR). Significant increases in AMPK phosphorylation and AMPK activity were observed when the cells were incubated with BBR. Activation of AMPK was also demonstrated by measuring the phosphorylation of acetyl-CoA carboxylase, a substrate of AMPK, correlated with a subsequent increase in fatty acid oxidation. All of these effects were abolished by the mitogen-activated protein kinase kinase inhibitor PD98059. Treatment of hyperlipidemic hamsters with BBR decreased plasma LDL cholesterol and strongly reduced fat storage in the liver. These findings indicate that BBR, in addition to upregulating the LDLR, inhibits lipid synthesis in human hepatocytes through the activation of AMPK. These effects could account for the strong reduction of plasma TGs observed with this drug in clinical trials.—Brusq, J-M., N. Ancellin, P. Grondin, R. Guillard, S. Martin, Y. Saintillan, and M. Issandou. **Inhibition of lipid synthesis through activation of AMP kinase: an additional mechanism for the hypolipidemic effects of berberine. *J. Lipid Res.* 2006. 47: 1281–1288.**

Supplementary key words low density lipoprotein cholesterol • PD98059 • 5-aminoimidazole-4-carboxamide 1-β-ribofuranoside • fatty acid oxidation • phosphorylation of acetyl-coenzyme A carboxylase

Coronary heart disease is the most important cause of morbidity and mortality in developed countries. Among the different risk factors, increased LDL cholesterol level has been identified as a major cause of coronary heart disease, and it has been extensively demonstrated in clinical trials that treatment of dyslipidemic patients with drugs that decrease LDL cholesterol levels significantly reduces the risk for coronary heart disease (1–4). Statins represent the major class of hypolipidemic drugs on the market. They act through the inhibition of HMG-CoA reductase, a pivotal enzyme in the cholesterol biosynthetic pathway, thus leading to a reduction of cholesterol concentration and a subsequent increase in expression of the low density lipoprotein receptor (LDLR), the main receptor involved in the hepatic clearance of LDL cholesterol (5–8).

Recently, ezetimibe, a new LDL cholesterol-lowering therapy described as a cholesterol absorption inhibitor, was developed, and it is now commercialized as a monotherapy or in combination with statins (9, 10). One interest of combination therapy is to reach the goals recommended by the National Cholesterol Education Program Adult Treatment Panel III (11) and to limit the potential side effects observed with high doses of statins (12). Clearly, the discovery of new drugs that could be developed in combination with statins is still of interest, especially compounds targeting other lipid fractions, such as HDL cholesterol and triglycerides (TGs), or other risk factors, such as type II diabetes and hypertension.

Berberine (BBR), an alkaloid isolated from the Chinese herb *Coptis chinensis*, has been widely used as a drug to treat gastrointestinal infections. Recently, BBR has been described as a new cholesterol-lowering drug (13). In this study, BBR treatment of 32 type IIb dyslipidemic patients led to a 25% decrease in LDL cholesterol and a 35% decrease in TGs. This LDL cholesterol-lowering effect was attributed to the activity of BBR on hepatic LDLR expression via a new mechanism distinct from that of statins. Indeed, the authors demonstrated that in a human hepatoma cell line (HepG2) as well as in hyperlipidemic hamsters, BBR upregulated the expression of LDLR

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through stabilization of its mRNA involving an extracellular regulated kinase (ERK)-dependent mechanism (13, 14). The authors concluded that it could be worth developing this new drug in combination therapy with statins. However, statin treatment of hypercholesterolemic patients with TG levels < 200 mg/dl often shows a more pronounced effect on LDL cholesterol than on TGs (15). Thus, even if the mechanism of action described by Kong et al. (13) is consistent with a LDL cholesterol-lowering effect, it is more difficult to explain the TG-lowering effect simply through upregulation of the LDLR. The aim of our study was to identify additional mechanisms by which BBR could exert its hypotriglyceridemic effect in humans. Using both in vitro and in vivo models, we demonstrated that BBR was able to inhibit cholesterol and TG synthesis through the activation of AMP-activated protein kinase (AMPK), leading to a decrease in hepatic fat content.

MATERIALS AND METHODS

Lipid synthesis

HepG2 cells obtained from the American Type Culture Collection were plated on 24-well plates. Cells were incubated for 6 h with vehicle or compounds at the indicated doses in the presence of 74 kBq of [^{14}C]acetate (Amersham), 18.5 kBq of [^{14}C]oleate (Perkin-Elmer), or 18.5 kBq of [^{14}C]glycerol (Perkin-Elmer) in RMPI 1640 medium supplemented with 2% lipoprotein-deficient serum. In some experiments, cells were preincubated for 30 min without or with 10 μM PD98059. At the end of the incubation, intracellular lipids were extracted with isopropanol and secreted lipids were extracted from supernatant by a mixture of cyclohexane-isopropanol (3:2, v/v). Both secreted and intracellular lipids were separated by thin-layer chromatography as described previously (16). Cholesterol and TGs were identified using purified standards, and the radioactivity associated with each individual lipid was quantified using a Phosphoscreen (Storm; Molecular Dynamics). Data are expressed as percentages of control values for cholesterol and TGs.

Measurements of Phosphorylated-acetyl-CoA carboxylase

HepG2 cells, plated on 96-well plates, were incubated for 6 h with the compounds at the indicated doses. Phosphorylation of acetyl-coenzyme A carboxylase (ACC) was performed using In Cell Western Protocol (Li-Cor Biosciences). Cells were fixed in 4% formaldehyde, permeabilized in PBS containing 0.1% Triton X-100, and then blocked with Li-Cor Odyssey Blocking buffer for 90 min. The antiphospho-ACC antibody (rabbit polyclonal IgG; Cell Signaling) was added to the cells overnight at 4°C. After several washes, a secondary antibody (IRDye 800 conjugated affinity purified anti-rabbit IgG; Rockland) was added for 1 h at room temperature. Fluorescence was quantified using the Odyssey Infrared Imaging system from Li-Cor Biosciences.

Measurements of AMPK phosphorylation and AMPK activity

HepG2 cells, plated on 24-well plates, were incubated for 6 h with the compounds at the indicated doses. At the end of the incubation, cells were lysed in Laemmli buffer containing phosphatase inhibitors (5 mM sodium pyrophosphate and 50 mM sodium fluoride). After SDS-PAGE and electrotransfer, AMPK was quantified using rabbit anti-α subunit (Cell Signaling) and AMPK threonine 172 phosphorylation was quantified using rabbit anti-pT172 antibody (Cell Signaling). Immunumodetection was performed with the Odyssey Procedure (Li-Cor Biosciences) using an IRDye800 coupled anti- rabbit IgG secondary antibody (Rockland).

For AMPK activity, treated HepG2 cells were lysed using 1% Triton X-100, and AMPK was immunoprecipited by Sepharose-coupled rabbit anti-AMPKα1 antibody (Abcam). AMPK activity was then determined by phosphorylation of the synthetic SAMS peptide, as described previously (17), in the presence or absence of 50 μM AMP.

Fatty acid oxidation assay

Fatty acid oxidation was measured in HepG2 cells plated on 96-well plates and incubated for 6 h with the compounds at the indicated doses and then challenged for 30 min with 3 kBq of [^{14}C]palmitate (50 μM in fatty acid-free albumin). Incubation was stopped with 5% perchloric acid, and acid-soluble metabolites were separated and measured by scintillation counting (18, 19).

In vivo activity

All experimental protocols were performed in accordance with the policies of the Institutional Animal Care and Use Committee. Animals were fed an appropriate diet and had free access to water. Male Syrian golden hamsters (Janvier) were fed for 2 weeks with a high-fat diet (0.12% cholesterol and 10% coconut oil). Then, animals were treated orally twice a day for 10 days with BBR at 100 mg/kg/day or vehicle (0.5% methylcellulose and 1% Tween 80, pH 7). At the end of the treatment, lipoproteins were analyzed as described (20). Quantification of lipid molecular species in the liver was performed by gas-liquid chromatography. Part of the liver was weighted and then crushed using an Ultra Turax in 1 ml of methanol and 5 mM EGTA (2:1, v/v). Aliquots corresponding to an equivalent of 0.5 mg of liver were evaporated, and then the protein pellets were dissolved in 0.5 ml of NaOH (0.1 M) overnight and measured with the Bio-Rad assay. Lipids corresponding to an equivalent of 1 mg of tissue were extracted in chloroform-methanol-water (2.5:2.5:2.1, v/v/v) in the presence of the internal standards: 6 μg of stigmasterol, 4 μg of diacylglycerol-1,3-dimyristoyl, 4 μg of cholesteryl heptadecanoate, and 6 μg of triheptadecanoyl glycerol. The chloroform phase was filtered through glass wool, evaporated to dryness, and dissolved in 20 μl of ethyl acetate. Two microliters of the lipid extract was analyzed by gas-liquid chromatography on the FOCUS Thermo Electron system using Zebron-1 Phenomenex fused silica capillary columns. Quantification of fatty acid methyl ester (FAME) molecular species in the liver was done by gas-liquid chromatography. Lipids corresponding to an equivalent of 1 mg of liver were extracted according to the method of Bligh and Dyer in the presence of nonadecanoic acid (2 μg) as an internal standard. The lipid extract was transmethylated with a mixture of acetyl chloride in methanol (1:20, v/v) for 1 h at 55°C. After evaporation to dryness, the FAMEs were extracted with 2 ml of petroleum ether and 2 ml of water. The organic phase was evaporated to dryness and dissolved in 20 μl of ethyl acetate. One microliter of FAME was analyzed by gas-liquid chromatography using Famewax RESTEK fused silica capillary columns.

RESULTS

BBR inhibits lipid synthesis and secretion in HepG2 cells

We first evaluated the impact of BBR on TG and cholesterol synthesis by incubating HepG2 cells for 6 h with

[^{14}C]acetate in the presence of increasing concentrations of BBR. As depicted in **Fig. 1A**, BBR dose-dependently inhibited both cholesterol and TG synthesis, leading to an IC_{50} of ~15 μg/ml for both lipids. Regarding the secreted fraction of lipids, Fig. 1B shows that the inhibition of lipid synthesis translates into a similar reduction of cholesterol and TG secretion, with IC_{50} of 10.4 and 5.8 μg/ml, respectively. These potencies are in total accordance with those obtained with BBR for LDLR upregulation (13). Moreover, these inhibitions were observed 6 h after the addition of the drug, and this time has been shown to be the optimal time point for LDLR upregulation (13). In addition, it was previously described that the effects of BBR on LDLR upregulation were dependent on the mitogen-activated protein kinase kinase (MAPK/ERK) cascade (13). As illustrated in Fig. 1C, D, preincubation of the cells with the MAPK inhibitor PD98059 at 10 μM blunted the BBR-mediated inhibition of cholesterol (63% vs. 97% of control) and TG (64% vs. 90% of control) synthesis. These results confirm that, as observed for LDLR upregulation, BBR effects on lipid synthesis are mediated by the MAPK/ERK pathway. Similar results were obtained in the secreted fraction (data not shown). In parallel, cells were incubated with 5-tetradecyloxy-2-furan-carboxylic acid (TOFA; at 2.5 μM), an ACC inhibitor (16). In contrast to BBR, TOFA specifically decreased TG synthesis (Fig. 1D) via a MAPK-independent pathway. In summary, we found that in HepG2 cells, BBR inhibits TG and cholesterol synthesis by a signaling pathway involving the MAPK/ERK cascade.

BBR activates AMPK

The AMPK has been proposed to act as a fuel gauge in mammalian cells. Among the large number of AMPK protein targets, HMG-CoA reductase and ACC are well identified (21, 22). These two enzymes, involved in cholesterol and fatty acids synthesis, are inactivated by AMPK-mediated phosphorylation, leading to cholesterol and TG synthesis inhibition. We decided to compare the effects of BBR with those induced by the AMPK activator 5-aminoimidazole-4-carboxamide 1-β-ribofuranoside (AICAR) on lipid synthesis. AICAR is phosphorylated in cytosol by adenine kinase and then is converted in AICA-ribotide, which mimics AMP and activates AMPK in various cells, including HepG2 (23). TG synthesis was determined using [^{14}C]acetate or [^{14}C]glycerol incorporation, whereas TG assembly was assessed by the incorporation of [^{14}C]oleate.

HepG2 cells were incubated for 6 h with [^{14}C]acetate, [^{14}C]glycerol, or [^{14}C]oleate in the presence of BBR (15 μg/ml) or AICAR (2 mM). As observed in **Fig. 2A**, BBR

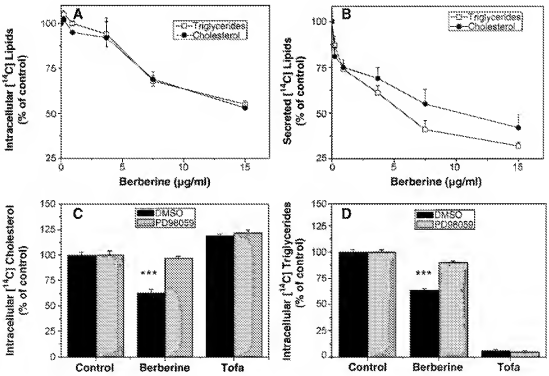

Fig. 1. Inhibition of lipid synthesis by berberine (BBR). A, B: HepG2 cells were incubated for 6 h in the absence or presence of BBR and labeled with [^{14}C]acetate. Intracellular (A) or secreted (B) ^{14}C-lipids were extracted and analyzed as indicated in Materials and Methods. Neosynthesized triglycerides (TGs; open squares) and cholesterol (closed circles) were quantified. Values are expressed as percentages of control and are means ± SEM (n = 5 from two independent experiments). C, D: HepG2 cells were preincubated for 30 min without (DMSO) or with 10 μM PD98059 and then further incubated for 6 h without (control) or with 10 μg/ml BBR or 2.5 μM 5-tetradecyloxy-2-furan-carboxylic acid (Tofa) and labeled with [^{14}C]acetate. Intracellular [^{14}C]cholesterol (C) and [^{14}C]-TGs (D) were quantified. Values are expressed as percentages of control and are means ± SEM (n = 11 for BBR, n = 5 for Tofa, from four and two independent experiments, respectively). *** $P < 0.001$.

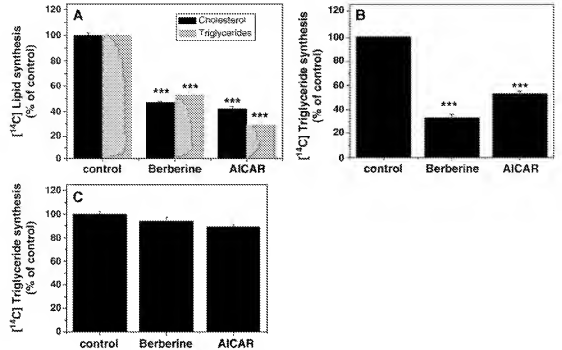

Fig. 2. Similar profiles induced by BBR and 5-aminoimidazole-4-carboxamide 1-β-ribofuranoside (AICAR) on lipid synthesis. HepG2 cells were incubated for 6 h in the absence (control) or presence of 15 μg/ml BBR or 2 mM AICAR and labeled with [^{14}C]acetate (A), [^{14}C]glycerol (B), or [^{14}C]oleate (C). Intracellular ^{14}C-lipids were extracted and analyzed as indicated in Materials and Methods. Values are expressed as percentages of control and are means ± SEM (n = 9, from three independent experiments). *** $P < 0.001$.

and AICAR inhibited [^{14}C]acetate incorporation into cholesterol by 53% and 58%, respectively, and into TG by 47% and 71%, respectively. When cells were incubated with [^{14}C]glycerol, BBR and AICAR inhibited TG synthesis by 67% and 47%, respectively (Fig. 2B). However, as shown in Fig. 2C, neither BBR nor AICAR prevented [^{14}C]oleate incorporation into TG, suggesting that TG assembly was not affected during the treatment. Moreover, these results suggest that BBR and AICAR induce similar profiles on lipid synthesis, suggesting that both compounds could regulate lipid metabolism by a common mechanism.

Therefore, we studied the possibility that BBR could activate AMPK. It is currently accepted that ACC phosphorylation levels into the cells represent a marker of AMPK activity (21). As shown in **Fig. 3A**, BBR dose-dependently increased ACC phosphorylation, with a half-maximal effect obtained at 2 μg/ml. Moreover, BBR-induced ACC phosphorylation was completely blocked in the presence of PD98059. In parallel, we showed that AICAR also increased ACC phosphorylation by 2.5-fold, an effect that was not affected by the presence of PD98059 (Fig. 3B).

AMPK activation leads to an increase in fatty acid oxidation, dependent on the inhibition of ACC by AMPK (24, 25). Figure 3C shows that BBR achieved a significant dose-dependent increase in fatty acid oxidation (half-maximal effect obtained at 10 μg/ml). As observed for the phosphorylation of ACC, this effect was totally inhibited in the presence of the MAPK inhibitor. In parallel, we showed that AICAR also increased fatty acid oxidation by 16-fold and that, as shown previously for ACC phosphorylation, this effect was not affected by the presence of PD98059 (Fig. 3D). We conclude that in HepG2 cells, BBR, but not AICAR, activates AMPK through a MAPK/ERK-dependent pathway, leading to the activation of fatty acid oxidation. This latter effect, associated with a decrease in TG synthesis, could explain the TG-lowering effect of BBR observed in patients and suggests pleiotropic effects of BBR on lipid metabolism.

Next, we evaluated the in cell activity of AMPK. After incubation of HepG2 cells with BBR, we monitored both the phosphorylation of the AMPK on threonine 172 and AMPK using specific antibodies. As shown in **Fig. 4A**, BBR significantly increased the phosphorylation of AMPK without altering the expression of AMPK (data not shown). Furthermore, preincubation of the cells with the MAPK inhibitor PD98059 at 10 μM suppressed the effects of BBR on AMPK phosphorylation.

To reinforce the activation of AMPK by BBR, HepG2 cells were treated with BBR and the enzyme was immunoprecipitated. AMPK activity was quantified using SAMS peptides (17). The assay was done in the presence or absence of 50 μM AMP (Fig. 4B). As expected for the AMPK, addition of AMP in the in vitro assay elicited a 2.3-fold increase in AMPK basal activity. Furthermore, cells treated with BBR exhibited an increase in AMPK activity, as demonstrated using the AMPK assay performed without or with AMP (1.7- and 1.6-fold, respectively, vs. nonstimulated cells).

Inhibition of lipid synthesis by BBR translates into a diminution of hepatic lipid and fatty acid content in vivo

Hamsters fed a diet enriched in cholesterol and fatty acids display a lipoprotein profile comparable to that of

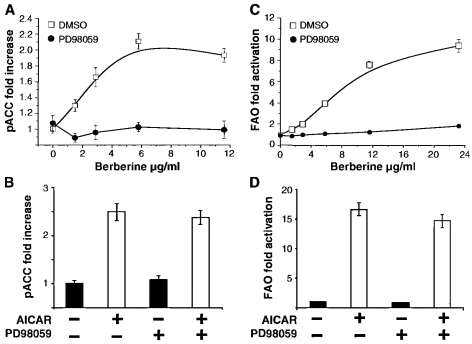

Fig. 3. Stimulation of acetyl-coenzyme A carboxylase (ACC) phosphorylation and fatty acid oxidation by BBR and AICAR. HepG2 cells were preincubated for 30 min without (DMSO) or with 10 μM PD98059 and then further incubated for 6 h with increasing doses of BBR or 2 mM AICAR. At the end of the incubation, phosphorylation of ACC (A, B) and fatty acid oxidation (C, D) were measured as indicated in Materials and Methods. Values are expressed as fold increase over untreated cells and are means ± SEM (n = 6, from two independent experiments).

humans (20). We investigated the effects of BBR on the hepatic lipid content in fat-fed hamsters. After 10 days of treatment with BBR (100 mg/kg/day), the serum LDL cholesterol fraction was significantly reduced by 39% compared with that in control animals. The HDL cholesterol fraction was unchanged (**Table 1**). The extent of the LDL cholesterol-lowering effect was comparable to that described previously (−42%) for the same dose of BBR in the same animal model (13).

In addition, BBR significantly decreased hepatic lipid content, with reductions of TG, cholesterol, and cholesteryl ester of 23, 27, and 41%, respectively. Interestingly, we also observed that hepatic fatty acid levels were significantly reduced by 16%. Thus, in addition to decreasing plasma LDL cholesterol, BBR induces a significant reduction of the hepatic fat content.

DISCUSSION

Kong et al. (13) described that BBR increased LDLR expression in HepG2 cells. They also demonstrated that this effect was not attributable to a transcriptional upregulation but rather to LDLR mRNA stabilization. Finally, they concluded that the LDL cholesterol-lowering effect (−25%) of BBR observed in hypercholesterolemic patients could be driven by LDLR upregulation. In addition, BBR in this patient population also displayed a robust and unexpected TG-lowering effect (−35%), in light of the mechanism of action described above. Statins, which also decrease LDL cholesterol levels through LDLR upregulation, have some significant TG-lowering effects, but in general the impact on TG is lower than that observed for LDL cholesterol (15). Thus, to better understand this additive beneficial TG-lowering effect, we decided to explore in more detail the effects of BBR on lipid synthesis and secretion by human hepatocytes. We found that in HepG2 cells, BBR strongly reduced TG and cholesterol synthesis and secretion. The IC_{50} values obtained for these lipid parameters are in accordance with the potency described for LDLR upregulation in this cellular model. In addition, as described for LDLR upregulation, we demonstrated here that lipid synthesis inhibition induced by BBR was also sensitive to a MEK inhibitor, suggesting that both phenomena, LDLR upregulation and lipid synthesis inhibition, are driven by a common pathway that requires MAPK/ERK activation.

Because this inhibition of TG synthesis could explain, at least in part, the TG-lowering effect observed in hypercholesterolemic patients treated for 3 months with BBR, we decided to dissect out the mechanism that could trigger this effect. We concluded that activation of AMPK induced by BBR could be responsible for this profile. Several reasons led us to postulate this assumption. Indeed, AMPK has been proposed to play a key role in the regulation of lipid metabolism (21). Two enzymes involved in cholesterol and fatty acid synthesis, HMG-CoA reductase and ACC, are considered the primary targets of

Fig. 4. Stimulation of AMP-activated protein kinase (AMPK) phosphorylation and AMPK activity by BBR. A: HepG2 cells were preincubated for 30 min without (DMSO) or with 10 μM PD98059 and then further incubated for 6 h with BBR at 24 μg/ml. Phosphorylation of AMPK and total cellular AMPK were measured by Western blot, as indicated in Materials and Methods. The ratio of phosphorylated AMPK (pAMPK) to AMPK signal was determined, and data are expressed as fold increase over untreated cells. Data are means ± SD of duplicates from one representative experiment. Similar results were obtained in another independent experiment. B: HepG2 cells were incubated for 6 h without or with BBR at 14 μg/ml. After immunoprecipitation, AMPK activity was quantified with or without AMP, as described in Materials and Methods. Values are expressed as fold increase over untreated cells and are means ± SEM (n = 5, from two independent experiments). * $P < 0.05$, *** $P < 0.001$.

AMPK and are phosphorylated by the enzyme, leading to cholesterol and TG synthesis inhibition (19). When compared with the well-known AMPK activator AICAR, BBR induced a similar profile on lipid synthesis, with inhibition of cholesterol and TG synthesis without any impact on TG assembly. These effects may be explained by data showing that BBR induces an increase in the level of phosphorylated ACC, considered a marker of AMPK activity. The half-maximal effect was obtained at 2 μg/ml, similar to the concentration required to inhibit lipid synthesis. In addition, as observed for lipid synthesis, the effect of BBR on the phosphorylation of ACC was completely abolished with the MEK inhibitor (PD98059). Phosphorylation of ACC led to a decrease in malonyl-CoA that relieves the inhibition of fatty acid oxidation (21). As observed for the phosphorylation of ACC, stimulation of fatty acid oxidation by BBR occurred in the same dose range and was also blunted by the MEK inhibitor. Furthermore, after BBR treatment, AMPK activity as well as phospho-AMPK levels increase inside the cells, as demonstrated by immunoprecipitation and Western blot experiments.

TABLE 1. BBR reduces plasma LDL cholesterol and lipid mass accumulation in liver of fatfed hamsters

	Plasma Lipids						Hepatic Lipids							
	VLDL Cholesterol		LDL Cholesterol		HDL Cholesterol		Cholesterol		Cholesteryl Ester		Triglycerides		Hepatic Fatty Acids	
Sample	g/l	% of control	g/l	% of control	g/l	% of control	nmol/mg	% of control	nmol/mg	% of control	nmol/mg	% of control	nmol/mg	% of control
Control	0.3 ± 0.1	100%	1.5 ± 0.3	100%	2.2 ± 0.3	100%	39.4 ± 7	100%	387 ± 120	100%	25.7 ± 1	100%	710 ± 101	100%
BBR	0.3 ± 0.1	100%	0.9 ± 0.2 ($P < 0.05$)	61%	2.2 ± 0.3	100%	28.8 ± 2 73% ($P < 0.05$)		229 ± 54	59% ($P < 0.05$)	19.8 ± 5 77% ($P < 0.1$)		596 ± 72	84% ($P < 0.1$)

BBR, berberine. Hamsters were orally dosed once a day during 10 days with vehicle (control) or with 50 mg/kg BBR. Plasma and hepatic lipids and fatty acids were quantified as indicated in Materials and Methods. Values are means ± SD of five animals.

Together, these data suggest that BBR induces the activation of AMPK, which translates into the phosphorylation of ACC, leading to the subsequent increase in fatty acid oxidation, decrease in fatty acid synthesis, and, finally, TG synthesis. Whether or not BBR activates AMPK by altering the AMP/ATP ratio or by an alternative mechanism is still unknown. Interestingly, we showed that this AMPK activation occurred through the MAPK/ERK pathway, thus confirming a putative link between this pathway and AMPK, as demonstrated previously (26). This finding could be of great interest because AMPK activation has been proposed as a valuable approach to target lipid disorders (22) and because antidiabetic drugs such as rosiglitazone and metformin have been described to act, at least partially, through AMPK activation (27). These results suggest that the TG-lowering effect of BBR observed in patients may be independent of LDLR upregulation, thus demonstrating pleiotropic effects of BBR.

To reinforce the physiological relevance of these cellular observations, we investigated the impact of BBR on hepatic lipid content in fat-fed hamsters. First, we confirmed the extent of the LDL cholesterol-lowering effect described previously for the same dose of BBR in the same animal model (13). In addition, we found that BBR treatment significantly decreased neutral lipid content as well as free fatty acid level. These findings suggest that the inhibition of lipid synthesis that we demonstrated in vitro on HepG2 cells translates into hepatic fat storage diminution in vivo. We speculate that this decrease in hepatic lipid content could lead to the improved liver function demonstrated by Kong et al. (13) in their clinical study. Indeed, BBR improved liver function in patients, based on alanine aminotransferase, aspartate aminotransferase, and γ-glutamyl transpeptidase activities; this property, in addition to circulating lipid effects, could be of great interest for combination therapies with statins or other lipid-modifying drugs.

In conclusion, we demonstrate that BBR, via AMPK activation, inhibits cholesterol and TG synthesis in hepatic cells. LDLR upregulation (13, 14), AMPK activation, and, finally, lipid synthesis inhibition are abolished when the MAPK/ERK pathway is blocked, suggesting that all of these events are linked to a similar mechanism of action. The precise mechanism by which BBR induces an ERK-dependent activation of AMPK remains to be elucidated. When administered to animal models, the effects of BBR on lipid homeostasis translate into a significant reduction in hepatic fat storage, confirming that BBR could represent a promising new approach to reduce LDL cholesterol and TGs as a monotherapy or in combination with other existing drugs.

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Topiramate stimulates glucose transport through AMP-activated protein kinase-mediated pathway in L6 skeletal muscle cells.

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The use of topiramate (TPM) in the treatment of binge-eating disorder, bulimia nervosa, and antipsychotic-induced weight gain has recently increased, however, the exact molecular basis for its effects on body weight reduction and improved glucose homeostasis, is yet to be elucidated. Here we investigated the effect and signaling pathway of TPM on glucose uptake in L6 rat skeletal muscle cells, which account for >70% of glucose disposal in the body. Intriguingly, we found that TPM (10 microM) stimulated the rate of glucose uptake up to twofold increase. And TPM-stimulated glucose transport was inhibited with the overexpression of dominant-negative form of AMP-activated protein kinase (AMPK), an important mediator in glucose transport, implicating that AMPK-mediated pathway is involved. The TPM-stimulated glucose transport was blocked by SB203580, a specific inhibitor of AMPK downstream mediator, p38 mitogen-activated protein kinase (MAPK) protein. LY294002, an inhibitor of phosphatidylinositol (PI) 3-kinase, which is another crucial mediator in independent glucose transport pathway, did not inhibit TPM-stimulated glucose transport. We also found that TPM increased the phosphorylation level of AMPK and p38 MAPK, whereas no effect on the activity of PI 3-kinase of TPM, when assessed by PI 3-kinase assay, was observed. These results together suggest that TPM stimulates glucose transport, not via PI 3-kinase mediated, but via AMPK-mediated pathway in skeletal muscle cells, thereby contributing to the body weight regulation and glucose homeostasis.

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MDA-MB-435 cells are derived from M14 melanoma cells--a loss for breast cancer, but a boon for melanoma research.

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BACKGROUND: The tissue of origin of the cell line MDA-MB-435 has been a matter of debate since analysis of DNA microarray data led Ross et al. (2000, Nat Genet 24 (3):227-235) to suggest they might be of melanocyte origin due to their similarity to melanoma cell lines. We have previously shown that MDA-MB-435 cells maintained in multiple laboratories are of common origin to those used by Ross et al. and concluded that MDA-MB-435 cells are not a representative model for breast cancer. We could not determine, however, whether the melanoma-like properties of the MDA-MB-435 cell line are the result of misclassification or due to transdifferention to a melanoma-like phenotype. METHODS: We used karyotype, comparative genomic hybridization (CGH), and microsatellite polymorphism analyses, combined with bioinformatics analysis of gene expression and single nucleotide polymorphism (SNP) data, to test the hypothesis that the MDA-MB-435 cell line is derived from the melanoma cell line M14. RESULTS: We show that the MDA-MB-435 and M14 cell lines are essentially identical with respect to cytogenetic characteristics as well as gene expression patterns and that the minor differences found can be explained by phenotypic and genotypic clonal drift. CONCLUSIONS: All currently available stocks of MDA-MB-435 cells are derived from the M14 melanoma cell line and can no longer be considered a model of breast cancer. These cells are still a valuable system for the study of cancer metastasis and the extensive literature using these cells since 1982 represent a valuable new resource for the melanoma research community.

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